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EFFECTS OF SOME ENZYMES INJECTED INTO THE SLIME MOLD PHYSARUM POLYCEPHALUM

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Enzymes are used in histochemistry on fixed and sectioned material as a means of identifying their substrates when these are stainable or absorb specifically in the ultraviolet. In the present study, enzymes have been injected into living protoplasm with the object not only of identifying their substrates, but also of showing how the latter are combined. It was assumed that the submicroscopic structure of living matter comprises macromolecules such as proteins, polysaccharides and nucleic acids whose organization is reflected in and largely responsible for both visible structure and certain physiological activities of cells. Thus, alterations in visible structure and certain physiological activities which are induced by the injection of enzymes may be regarded as due to their action on some submicroscopically organized structure in which the specific substrate plays an important part.

A series of enzymes whose substrates are proteins and sugar or nucleic-acid-containing substances was injected by micropipette into the protoplasm of the slime mold *Physarum polycephalum*. Cultures of this plasmodium are a very favorable material for this type of study for several reasons. Because of their size the disturbance of the protoplasm during the injection can be kept down to a minimum. Their character as syncytia permits the enzyme solutions to come into contact with numerous parts of the protoplasm which may be in different physiological states. Physiological states

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which may be observed with the microscope include the formation of pseudopodia and of regression regions, as well as the characteristic to-and-fro streaming of the protoplasm. The material is highly variable and responsive to many stimuli. As the interpretation of visible alterations in physiological state presupposes a great familarity with the reaction pattern of the organism, a short account of the structure of the mold and its normal behavior has been given. These observations are limited largely to those features which have been found to be of importance for the present work.

Seven different enzymes were used for the experiments. Each one had its specific effect upon protoplasmic structures, such as hyaline layer, plasmasol, plasmagel, channel walls, etc., and also produced consistently characteristic modification of protoplasmic streaming. By an analysis of the experimental results it could be inferred that the hyaline layer contains simple proteins, while the plasmagel contains complexes of proteins with carbohydrate and nucelic acid. The results reveal a new aspect of the enzymic mechanisms involved in the reversible gel/sol transformation which is an important part of the protoplasmic streaming, and of pseudopod formation and locomotion.

MATERIALS AND METHODS

Plasmodia of the slime mold *Physarum polycephalum* were grown by the method of Camp ('36) on moist filter paper by feeding them rolled oats. Small portions of protoplasm of about 10 mg were transferred from the stock cultures to moist cover slips on which they grow and spread out for many hours. The inverted cover slips were placed over the moist chamber for observation and microinjection. Microinjections were made into strong branches of the plasmodium in the following way: A slight pressure was built up in the injecting system of the apparatus and the micropipette plunged into the protoplasm. This reduces the incidence of clogging at the tip which tends to occur when it touches the cytoplasm. The amount of liquid injected was about one-thirtieth of the whole mass of protoplasm present in the experimental plasmodium. No attempt was made to inject precise quantities of solution for reasons which will be indicated later. When the liquid entered the plasmasol the normal yellow-brown color became a pale yellow and this spread within a few seconds throughout many of the channels of the culture. This decoloration was a good index that the liquid had really entered the protoplasm.

After a few seconds the following conditions became established: In the regions away from the site of injection the liquid mixed readily with the plasmasol; in the regions nearer to it coagula were formed in the channels through which the liquid had passed; at the point of entrance a more or less circumscribed area of decay was indicated by the white color and sometimes surrounded by a thick coagulum wall. The coagula in the channels were usually resorbed within minutes, in rare cases within hours, whereas decay regions were often not resorbed. The coagula caused by different injection liquids behaved differently, as will be pointed out later. The more pronounced the formation of coagula and decay regions, the more detrimental an injection was considered to be. The damage is considered to be only slightly harmful if small coagula (or none at all)were produced and if these were readily reabsorbed.

It can be assumed that those substances which mixed immediately with the plasmasol without any formation of coagula were best able to produce specific effects. It also may be assumed that parts of the injected solution were trapped within the coagula and that the active substance was released into the protoplasmic stream when reabsorption of the coagula took place. Finally it may be assumed that substances contained within the disintegrating areas were largely retained and did not diffuse readily into the surrounding living protoplasm. This could be attributed to an instantaneous formation of a cell membrane, which would tend to retard or prevent the diffusion of substances of large molecular weight, such as enzymes. As the proportion of the volume of protoplasm affected by the injected solution in these three ways is undoubtedly very variable and unknown it was felt that it would be of no advantage to inject precise amounts of liquid.

The fact that a considerable portion of the plasmodium may coagulate or even disintegrate does not of itself invalidate an experiment. The plasmodium of a slime mold is not a highly organized and integrated individual whose function as an organism is impaired by the loss of one of its parts. After an injection, the cytoplasm of those parts of a culture which had not been immediately injured resumed its flow, usually in a few seconds. Sometimes it happened that two parts of a culture were separated by a decay area — each part then behaved as if it were independent.

All of the injected enzymes, with one exception, were commercial preparations. Papain and pectinase were crude products from the Nutritional Biochemicals Corporation. Trypsin, pepsin, and hyaluronidase were crystalline preparations obtained from Armour Laboratories. Collagenase was kindly furnished by Dr. E. Bidwell, formerly of the Wellcome Physiological Research Laboratory, England. Ribonuclease was a salt-free product prepared according to directions of Kunitz. No attempt was made to determine their degree of purity or the enzymatic activity of the solutions. All solutions were made up in double distilled water immediately before use, and were never older than 4 hours. The optimum concentration, ascertained for each enzyme by trial and error, will be indicated in every case.

The effects which have been recorded are those which could be observed as visible modifications of the structure and activity of plasmodia.

Every report on the activity of an injected enzyme solution is based on about 10 experiments, whose common features are noted. It goes without saying that there were variations. These could be expected in a material which is subjected to many influences and which is as unpredictable in its behavior as slime molds.

OBSERVATIONS AND RESULTS

I. General features of structure and activity

1. Structure. In every healthy plasmodium it is easy to distinguish two parts: (1) A gelated non-motile portion consisting of a network of vein-like channels, and (2) the solvated contents of this network which flow forward and backward continually. The advancing anterior margin, the pseudopod, usually presents an irregular or lobed appearance. The lobes resemble closely the advancing pseudopods in *Amoeba.* Because of the structural similarity of *Amoeba* and *Physarum* it is possible to transfer to a large extent the terminology which has been developed in the study of the former to the latter.

The pseudopods are often thin enough to permit observation with the high power lens of the microscope. A very thin surface layer or membrane can be seen which completely encloses the plasmodium. This membrane is homologous with the plasmalemma in *Amoeba* (Mast, '26). Beneath the limiting membrane, especially prominent at the anterior margin of active pseudopods, is a layer of clear hyaline plasm showing a faint bluish-grey color. Sometimes it happens that a pseudopod is spread out as a thin hyaline sheet with no granular plasm visible. Such areas may persist for varying lengths of time without visible change. During regression there may be perforations within this sheet — when these enlarge, a network of small veins is formed between them. Clear hyaline plasm can be found also in certain very thin channels or veins, which contract and relax periodically while contraction waves move slowly along them to drive out plasmasol with formed bodies. This layer is extremely thin in thicker veins. The hyaline layer seems to be homologous with the ectoplasm of other authors. Its most prominent characteristics are the gelled condition and the relative freedom from formed bodies.

The bulk of the gelated protoplasm or plasmagel contains numerous formed bodies which include granules, particles, vacuoles and nuclei. The plasmagel has a relatively tougher consistency and is organized to form an anastomosing network of channels or veins. It may be considered as the "skeleton" of the plasmodium. Camp ('37) believes it to be the living portion proper. Contained in the channels we find the liquid or solated portion of the protoplasm, referred to as plasmasol. It is believed to contain the same types of formed inclusions as the plasmagel from which it differs solely by its far lower viscosity. Camp ('37) came to the conclusions that the viscosity of a plasmodium varies from place to place with changing internal and external conditions, and that alterations from gel to sol and from sol to gel take place constantly in the cytoplasm. When the surface is punctured with a microneedle protoplasm flows out from pseudopods which had been strongly motile, while in motionless regions it oozes out. In the latter case highly viscous protoplasm clings to the needle when it is withdrawn. When motionless protoplasm is pressed with a blunt needle no changes in spatial relations of protoplasmic constituents can be observed, indicating that it has a high viscosity. On the other hand particles are easily displaced in the more liquid plasma.

When a needle is pressed lightly against some part or introduced into a channel, the plasmasol for some distance gelates. Without further disturbance this gelled plasm will gradually solate and begin to move again. The degree of gelation and the time required for solation depends upon the nature and vehemence of the stimulus applied. All these observations suggest that the viscosity of protoplasm varies from time to time even in the same region, and in different regions at the same time.

The plasmasol is distributed throughout the gelatinous portion in the intricate network of channels and veins. There are various degrees of viscosity of the plasmasol. One part is actually flowing back and forth in well-established channels. This portion has the least degree of viscosity. Another part is more viscous and is "stored" in certain parts of the culture. This is relatively immobile and seems to merge and blend imperceptibly with the more solid plasmagel. It will be referred to as ''reserve plasm.'' The more actively a culture flows over the substratum, the larger is the portion of the plasmasol which is in actual motion. The maze of small channels contained in the plasmagel appears then more clearly and makes it look porous or spongy. We shall refer to this condition by saying the reserve plasm has been mobilized.

2. Activity: Protoplasmic streaming. The activity to be considered here is known in its entirety as protoplasmic streaming. It includes the rhythmic forward and backward flow which is characteristic of slime molds and a slow creeping over the substrate. The rhythmic flow is characterized by the flow of plasmasol in one direction, the gradual cessation of flow and the reversal of its course. The time between each period varies from 15 to 55 seconds. The contraction of the gelated portion of the cytoplasm can be observed in small channels. When the plasmasol streams away from a pseudopod it can be seen to shrink strongly, especially when it is not yet firmly attached to the substratum and is floating freely in the water. The pseudopods will expand again with a new incoming wave of plasmasol. These rhythmic expansions and contractions will be termed pulsation.

When a small portion of protoplasm is cut off from the stock culture and transferred to a cover slip, all movement stops for a while. If the trauma is great the return of protoplasmic streaming is greatly delayed, sometimes for an hour or more. In this case movement begins in very small districts, more or less simultaneously, but without much coordination. In time coordination is established between adjoining districts, and connecting channels are formed. This development gradually spreads over the new culture. Pseudopods are extended and forward flow begun only after a kind of uniform rhythm has been established throughout the whole mass of protoplasm. In larger cultures with numerous pseudopods coordination of rhythmic flow is not perfect for all of its parts, and there is variety in direction and velocity of flow. Smaller parts, however, such as single pseudopods, are usually in perfect coordination. The velocity of rhythmic flow is variable depending on the viscosity of the plasmasol, width of the channels, coordination of the pulsations and perhaps other factors.

Forward flow is the process whereby the whole plasmodium creeps slowly over the substratum. It can be broken down into three elementary processes: The advancement proper, the recess at the rear, and the displacement of material from the rear end to the anterior margin.

At certain points of the advancing margin protrusions of the hyaline layer are formed which are known as hyaline caps. Their size, position and shape vary continuously. At times they appear as caps extending across the face of a broad branch; more often they are not so wide and more than one may be present. There is a certain uniformity in the way they are formed. With eruptive speed a finger-like or blister-like protrusion of the hyaline plasm appears as though some limiting membrane were weakened locally, giving way to an inward pressure of the protoplasm. At these points the hyaline plasm solvates and is pushed forward to gelate again after a few seconds. At the base of the caps the granular plasm can be seen forming a distinct demarcation line. After a while, usually with the advent of a new wave of plasmasol, the protrusions enlarge and are filled with formed elements. In this state they are referred to as pseudopodial lobes. Finally the formed bodies mix with the hyaline plasm completing the formation of a mature lobe. The development of both apical and lateral lobes may subsequently lead to a change in their size and shape.

Differences in size and shape of advancing pseudopods may be observed. The simplest form is a semicircular cap terminating a long extending branch. It advances slowly and evenly while the margin remains a smooth semicircle at all times. Let us call this a simple tip; it is regarded as a sign of weak activity. More often pseudopods develop into a widely spread fan-like structure terminating in a multilobed margin which results from a diversified and simultaneous production of many protrusions. The region behind the advancing margin is usually thick and tapers down to a thin layer, the hyaline sheet, with its numerous channels. This whole structure will be called a compound pseudopod; its presence is a sign of vigorous activity. There are transitions from one type of pseudopod to the other. After a piece of protoplasm has been transferred to a moist cover slip the new culture usually starts out with a few simple pseudopods, which rapidly develop into the compound form. After several hours of vigorous activity the simple form may reappear. The transition may occur several times and may take place within 10 minutes. When material for a new subculture is taken from a vigorously advancing pseudopod of the stock culture and care is taken not to injure the material, the new culture will soon produce numerous compound pseudopods. When a subculture is started from a part of the stock culture which was sluggishly moving or stationary the new culture persists with simple pseudopods, or none.

No visible change can be perceived in the plasmalemma during advancement, but it is evident that it suffers a great deal of stretching with the formation of lobes. This stretching can, of course, not go on forever, there must be a deposition of material in order to account for the immense increase in size at points of forward movement. In the case of *Amoeba* the possibility had been discussed that the plasmalemma might roll over the whole animal like a rolling sac. This type of mechanism seems highly improbable for plasmodia with their tremendous change in shape and surface area.

In every advancing plasmodium there is always an area in which regression takes place. Such an area may be formed under adverse conditions such as lack of moisture, food in a different direction, and others. The first sign of regression may be noted when the incoming plasma wave is far weaker than the outflowing wave. The contraction in such a region is exceptionally strong and is only partially relaxed. The outlines of a regression region lose their round and smooth appearance and often look shriveled. Delicate and tenuous projections may appear in the plasmalemma; these are possibly folds in the latter caused by the loss of surface area. Frequently small bubble-like protrusions appear every time a protoplasmic wave streams into a receding branch. These protrusions develop exactly in the same way here as at the advancing margin of a pseudopod, the eruptive suddenness being the most prominent feature. There is, however, this difference, that protrusions at the receding end are immediately withdrawn and do not give rise to further advancement. In other cases the plasmalemma is broken down first and all that is left behind is a clearly exposed network of channels. Their plasmagel is gradually broken down around the numerous particles which are left behind.

The fundamental process underlying regression is known as solation and is thought to be a simple change in the physical state of the protoplasm. Many authors believe that hydration plays the most important part. It should also be borne in mind that "gelated" protoplasm is conceived as a structural lattice of long protein chains, one function of which is contraction and relaxation. These are believed to be present until shortly before solation takes place, as evidenced by the vehement contractions at regressing branches. Solation, consequently, must be related to or include the degradation of these proteins, probably by proteolytic enzymes. It is not known how far the breakdown goes; that is, whether it stops at relatively highly complex elements, or whether it goes further to small peptides or even amino acids. The results of the degradation are evidently those "prestructural elements" postulated by Winer and Moore ('41) which are destined to serve as raw material at the advancing margin of the plasmodium. An indication of how far the breakdown goes can be found in the viscosity of the plasmasol. The breakdown particles would seem to be smaller in vigorously streaming cultures, for two reasons: (1) The velocity of flow is high, and (2) when the surface of a pseudopod is punctured protoplasm leaks out readily and mixes freely with the surrounding medium without gelation. The breakdown products would seem to be longer in sluggish cultures: (1) Flow is slow and (2) when a branch is punctured little (if any) plasmasol flows out, and this gelates almost immediately.

The third process necessary for forward flow is the displacement of solated material from areas of regression to regions of advancement. This is managed by a change in the nature of the rhythmic flow, a greater amount of material being displaced in the advancing direction. This phase of cytoplasmic flow has been discussed already.

In summary, the active type of colony lavishly produces hyaline protrusions resulting in pseudopodial lobes and compound pseudopods. Forward movement is relatively rapid, as is the rhythmic flow of the plasmasol. The viscosity of the latter is low, and a high percentage is mobilized and takes part in the active movement, which means that little reserve plasma is present. Less active colonies are characterized by few hyaline protrusions, simple pseudopods, sluggish rhythmic flow, high viscosity of the plasmasol, a low percentage of it being in motion, with little if any forward movement. It should be emphasized that all the characteristics of either series normally occur together. They are, of course, more pronounced in extreme cases and there are all degrees of transition between the extremes. The controlling factors will be discussed below.

II. Effects of injected enzymes

1. Effects of the medium and of inorganic ions. The following is a description of some of the visible effects of injecting doubly distilled water and water containing inorganic ions.

Wherever the water mixed with the plasmalsol this assumed a pale yellow color. In areas of strong penetration coagula were formed and at the point of entrance the characteristic decay region appeared. These injuries were, however, not severe. In some cases when the injection had been made slowly enough or when a culture was in the state of very active streaming, no coagula were noticed. In most cases all of the coagula, even the decay regions, were resorbed within the next 10 minutes. The decay regions were rather small even with heavy injections. There were also some effects on the rate of flow. Rhythmic flow usually stopped for two or three seconds, a characteristic reaction to all strong stimuli applied to a plasmodium. It was resumed immediately in all of the non-injected portions of the cultures and spread little by little to areas into which liquid had penetrated. In some cases, especially when coagula filled a main branch, flow was slow at first, normal velocity being re-established as a rule 5 minutes afterwards. Later there was no increase in the rate of flow, nor was there any change in the rate of forward movement or in the state of activity. It was always found that about 15 minutes after the injection the previous behavior of the culture had returned, with no persistence of the residual effects of the early reaction.

In order to test for the activity of inorganic ions upon the slime mold, Chambers' solution was injected. This was proposed by Chambers ('49) as being rather innocuous when injected into slime molds. It contains 0.120 M KCl, 0.013 M NaCl and 0.003 M $CaCl_2$. The following effects were observed. Even after a large dose rhythmic flow stopped only for a few secconds. Slight coagula were formed, or none. The region which with other types of fluids developed into a decay region was reabsorbed within 10 minutes without the characteristic shift of color toward white. No membrane was formed around this area. There was a pronounced effect on the hyaline layer in that numerous finger-like projectons were extruded for about one minute. The formation of pseudopodial lobes was frequently accelerated with an enhancement of forward movement. The rate of rhythmic flow increased shortly after the injection when the coagula had been reabsorbed and the system of channels cleared. This speeding up of rhythmic flow together with the enhanced formation of pseudopodial lobes gave the impression that protoplasmic streaming was greatly activated. There was, however, no apparent mobilization of reserve plasm. If the major portion of the injected liquid entered into one pseudopod this became a regression region within 10 minutes even if it were actively flowing forward before. The effects of activation lasted for about one hour after which time the cultures entered into a condition of sluggish movement. Calcium-free Chambers' solution had similar but somewhat more marked effects.

A 0.02 M solution of $CaCl_2$ acted in the opposite way. Very little of the injected liquid really entered the protoplasm, because the plasmasol gelated almost instantly with the formation of a thick membrane. The point of entrance was always marked by a severe decay region. Only small portions of the grey coagula which formed were resorbed; the decay region persisted. The small amount of solution which had penetrated beyond these regions caused an almost immediate cessation of rhythmic flow which was resumed slowly after 10 to 15 minutes, to return to normal after about an hour. Forward movement stopped completely.

This series of experiments demonstrates that inorganic ions have a great effect on the structure and activity of slime mold cultures, far beyond those produced by distilled water. Other substances, such as glucose and sucrose also affected the plasmodia unfavorably. Consequently it was decided to use pure water in all following experiments with enzymes. The effects of pure water can easily be taken into account, they are short-lasting, and have no further influence upon the state of activity of the culture. It is assumed that any departure from these effects after the injection of an enzyme solution is due to the activity of the particular enzyme.

2. Proteolytic enzymes (see table 1). (a) Papain. The concentrations which gave the best results were from 0.2 to 0.5% by weight of the crude commercial product. Higher concentrations showed essentially the same effects but were far more harmful in that they produced a very severe decay region and often stopped forward movement. The immediate effects of an injection of papain into the protoplasm were as follows: Coagula and decay regions were formed and then readily resorbed, usually within the next 10 minutes. Proto-

TABLE 1

Effects of enzymes and other substances following their injection into Physarum

	FORWARD FLOW	RHYTHMIC FLOW	HYALINE PROTRUSIONS	RESERVE PLASM	PULSATION	COAGULA ABSORPTION	DECAY REGION ABSORPTION	REGRESSION REGION
Water	chiefly unaffected	chiefly unaffected	not produced	unaffected	unaffected	fast	fast	not initiated
Chambers' solution	accelerated	accelerated	some produced	unaffected	unaffected	fast	fast	initiated
0.02 M $CaCl_2$	stopped	strongly impaired				very slow	none	
Papain	strongly accelerated	strongly accelerated	many produced	mobilized	more vigorous	fair	fair	not initiated
Trypsin	prevented	impaired	many produced	mobilized	weakened	slow	slow	not initiated
Pepsin	prevented or accelerated	accelerated	sometimes produced	partially mobilized	more vigorous	very slow	none	not initiated
Collagenase	suppressed	stopped	sometimes produced	mobilized	weakened	very slow	very slow	not initiated
Pectinase	suppressed	sometimes accelerated	suppressed	mobilized	not affected	fair	fair	initiated
Hyaluronidase	suppressed	accelerated	suppressed		not affected	fair	fair	initiated
Ribonuclease	suppressed	suppressed	suppressed	mobilized	weakened	none	none	initiated

plasmic streaming was resumed a few seconds after the injection. Sometimes it was somewhat slower within the following 5 minutes. After that period there was an acceleration of streaming which sometimes became unusually fast. The reversal of direction was sudden.

The most striking effect appeared in the hyaline layer, from 5 to 10 minutes after injection. At the anterior margin, sometimes also along the large channels, numerous finger-like and blister-like protrusions of the hyaline layer appeared. They had, as a rule, the same aspect as normal hyaline protrusions and were, like these, thrown out with explosive vehemence, but they were far more numerous than before the injection. They were withdrawn after 5 to 10 minutes. Despite resumption of a normal appearance the hyaline layer seemed to be affected for a long time, because pseudopodial lobes were more numerous and forward flow was more active. Most of the reserve plasm became mobilized so that the internal structure of the plasmagel could be perceived more clearly. The transformation of the hyaline sheet into small channels took place more rapidly. The rhythmic pulsations of the pseudopods became strikingly vehement.

All these phenomena taken together seemed to indicate that there was an activation of the normal process of protoplasmic streaming. The aspect of an injected colony was by no means abnormal, it looked exactly like a normal culture in the state of vigorous activity. This is borne out by the observation that the effects were most striking in colonies which had been in a state of sluggish movement for several hours and which normally would not have shown vigorous activity of the kind described. This transformation of a slow plasmodium into a vigorously streaming culture could take place within 10 minutes. The effects of injections lasted for about an hour after which there was regularly a noticeable reduction in activity.

(b) Trypsin. The optimal concentration was found to be of .002% by weight, with a trace of enterokinase added. There was a very pronounced tendency to coagulate and cytolyse the protoplasm which prevented a thorough and easy pene-

tration of the solution into the living parts of the cultures. The coagula became most evident after about 5 minutes, and retained the natural color of the protoplasm. They were resorbed very slowly and sometimes remnants of coagula could be detected hours later. The behavior of the decay regions was unique. They were extraordinarily large, so that a major portion of the liquid was trapped in them. Their color was first white, but slowly they resumed the normal brownish-yellow color of the uninjured protoplasm. They were invaded by small pseudopodial lobes which absorbed them little by little. Small areas were absorbed after half an hour; in larger ones the process took an hour or more. Absorption was more rapid in active colonies.

It may be assumed that a large portion of the enzyme was trapped in these areas. This would explain the fact that the effects which will be described below, were almost completely localized to the injected area or its immediate vicinity, while other parts of the colony might show a normal behavior. During the process of recovery some portions of the enzyme solution probably got into the streaming plasmasol and were carried to other parts. The timing of absorption influenced also the period over which the effects could be observed.

Usually rhythmic flow did not stop immediately after injection; only after a few minutes did it slow down, and sometimes stop. Flow returned within 5 to 10 minutes and became normal not earlier than 15 minutes afterwards. There was also a noticeable weakening in the rate of pulsation in the injected pseudopod. It should be emphasized that weak contractions and weak flow could be observed in channels which did not show any sign of coagulation.

Numerous hyaline protrusions appeared at the advancing margin of the pseudopod into which the injected liquid penetrated. They were usually of the finger-like type and originated with the usual eruptive speed. In some cases they were filled with granular material and assumed the shape of small, round blisters. The phenomenon became evident within the first few minutes, provided the margin of a pseudopod was near enough to the decay region. The production of these protrusions might be explained by a temporary solation of areas of the hyaline layer. It is the extreme opposite of the strong coagulating activity of the same enzyme on the plasmasol. Although there were numerous hyaline protrusions at the advancing margin there was little or no forward flow to be observed. This was resumed only after the effects of the injections had ceased. There was also a considerable mobilization of the reserve plasm.

(c) Pepsin. The optimal concentration was found to be 0.2%. The solution was used immediately after preparation to avoid deterioration. Coagula and white decay regions were formed rapidly. The former were absorbed very slowly, many of them persisting after an hour; some of them turned black and were not reabsorbed. Decay regions were never reabsorbed. When the enzyme came into contact with the hyaline layer numerous finger-like protrusions and small pseudopodial lobes were produced. They usually appeared at the advancing margin of the nearest pseudopod. In some cases it happened that the injected liquid spread throughout the whole culture. In these cases protrusions could be observed everywhere, even at the walls of main branches which rarely produce such protrusions under ordinary circumstances. Projections appeared from a few seconds after the injection to 20 minutes afterwards depending upon whether the enzyme could exercise its effect directly because of rapid spreading or slowly after it had become incorporated from the coagula. The new formations persisted for a few minutes. Although they deviated somewhat in appearance from normal pseudopodial lobes, they developed sometimes into mature lobes. In some cases there was only a broadening of the hyaline layer.

Rhythmic flow usually stopped shortly after the injection, and was resumed after three to 10 minutes, depending upon the amount of coagulum present. Flow in the rest of the cultures continued for a period of 5 to 10 minutes as usual. After this time, when presumably the enzyme had mixed with the plasmasol, flow slowed down for a few minutes, and then became accelerated, sometimes markedly. It can be assumed that there was a degradation of molecules which resulted in decrease of viscosity, which in turn was reflected in the increased rate of flow. The effect on forward movement depended upon the state of the injected colony. If the plasmodium were in a state of incipient activity, this was increased by the injection. In this case many of the hyaline protrusions developed into true pseudopodial lobes with further advancement in flow. These cultures showed most prominently the increase in the rate of rhythmic flow. In cultures which were already in a very active state at the time of injection the rhythmic flow was slowed down and the forward movement impaired for a period of about 20 minutes. After this time both types of movement returned to normal.

There was always a vehement pulsation in the pseudopods except when their plasm was actually in a state of coagulation, despite a slight mobilization of the reserve plasm. The period over which the effects could be observed depended on the amount of liquid injected and the condition of the culture. Strong and active cultures resumed their previous behavior after about half an hour. The increase in activity lasted usually more than an hour.

3. Collagenase (see table 1). The optimal concentration was 0.2%. At first sight the injection seemed to be quite innocuous. There was only a slight decoloration of the injected plasmasol with apparently little coagulation so that the liquid sometimes penetrated even small veins and branches. Flow was resumed after a few seconds often with the same velocity as before the injection. Only after about 5 minutes a steady decrease in the rate of flow could be observed until it finally stopped in the injected areas, sometimes for as long as 20 minutes, after which time it was resumed, though at a low rate for the next hour. In uninjected areas flow was not affected for 20 to 30 minutes after the injection; then it slowed down, although not as markedly or for as long as in the injected field.

The most conspicuous effect in the injected region of most specimens was the formation of a broad hyaline layer in the nearest pseudopod, often with numerous protrusions. When present, this phenomenon lasted for a few minutes. Its absence may have been due to the possibility that the liquid did not advance as far as the hyaline layer.

Although there was little formation of coagula at the beginning they became visible after 5 to 10 minutes by reason of their greyish color. At this time also decay areas became established. Resorption of both was extremely slow, extending sometimes over hours. It may be assumed that absorption had begun earlier and that enzyme molecules which had been released by this process were set free to mix with the flowing plasmasol and be carried to other parts of the plasmodium where they exercised their activity. Together with the cessation of rhythmic flow there was also a noticeable weakening of rhythmic pulsations in the injected pseudopods. This weakening appeared later also in other parts. Forward flow was suppressed for a period of about two hours. In quite a few cases liquid gathered slowly in one or more large white blisters of the plasmalemma which were eventually pinched off after several hours. In all the injected cultures a very striking mobilization of the reserve plasm occurred, usually after 10 to 15 minutes, and spread eventually over the whole plasmodium.

4. Polysaccharases (see table 1). (a) Pectinase. The optimal concentration was a 2% solution. The injections proved to be rather innocuous as small coagula were formed and a relatively large amount of the injected liquid was absorbed immediately. This was particularly true in cultures with rapid flow. Resorption of coagula was completed in one hour, the same being true for decay regions when present. Flowing was resumed a few seconds after injection, but it slowed down within the following minutes. After 10 minutes its normal rate was reestablished. In cases in which flow had been very active before the injection it became as rapid as before, sometimes even more rapid. In a few cases an extraordinary increase in the rate of flow was noticed. This occurred particularly when flow was sluggish before the experiment so that inactive cultures were transformed into rapidly streaming plasmodia. There was always a sharp increase in the amount of liquid plasmasol which could be ascertained by the usual signs of active plasmodia: They flattened out and the interior structure of microscopic channels became evident which means that reserve plasm had disappeared from thence. Areas which had received a major portion of the injection became regression districts. In these the plasmagel was gradually liquified presumably to be displaced to other pseudopods. Curiously enough, the other pseudopods very seldom showed traces of forward flow; instead the liquid material accumulated in them and gave them a swollen appearance. Hyaline caps and lobes disappeared and their margins smoothed out, as though yielding to an internal pressure. These changes took place in cultures which otherwise showed clearly all other signs of active streaming. The influence of the injection became evident usually at about 20 minutes, and ceased after about one hour's time.

(b) Hyaluronidase. The optimal concentration was 2% by weight. The injections proved to be relatively innocuous. In some cases no coagula were formed at all, the injected liquid being absorbed immediately. In other cases slight clotting action could be noticed, the coagula being resorbed within 10 to 30 minutes. Decay regions at the site of entrance disappeared within an hour or so, so that after this period there was seldom a trace left of a preceding injection. Rhythmic flow was resumed either immediately or within one or two minutes, and it recovered speed within the next 5 to 10 minutes. This was followed by a sharp increase of velocity beyond that shown before injection. This accelerated flow lasted usually for an hour, sometimes longer.

Forward flow was either impaired or suppressed altogether. In active cultures with multilobed, profusely spread pseudopods which had a good hyaline cap production, these pseudopods were withdrawn within the next 20 to 30 minutes and reduced to a blunt stagnant branch. The size of the pseudopods gradually diminished, their margins became smooth, and the protoplasm broken down. Yet while this was taking place no other portion of the culture advanced as normally happens in regular colonies. All of the liquid material was simply floating back and forth, and this with a considerable speed, indicating that the viscosity of the plasmasol was evidently low. All of the remaining vestigial pseudopods could be seen contracting and expanding vigorously. There was, then, the paradoxical condition of a vigorous rhythmic flow with an increase in the amount of plasmasol unaccompanied by advancing flow. If sometimes a bulge were formed, this seemed to be due more to a yielding to internal pressure than to be true lobe formation. The central branches became broader and thicker since much of the material accumulated there. After about half an hour the colonies gave an impression of stagnation, except for the vigorous rhythmic flow. In contrast with the above, cultures which had not been as active at the time of injection showed these effects to a lesser degree. In these also there was no forward movement. After a small dose of injection fluid flow was resumed after an hour's period, while those which had received more remained stagnant for several hours.

5. Ribonuclease (see table 1). The optimal concentration was found to be 0.1%. The injected liquid was quickly absorbed with only a slight decoloration in the injected plasmasol, no sign of clot formation and rarely a decay region. After about 5 minutes there were usually no residual signs of the injection. After 15 to 20 minutes a kind of decay region became visible as a greyish tint of the plasmagel. Closer examination revealed that there was no coagulation as yet in these greyish portions; there was a typical microscopic network with rhythmic flow. Later, when the injected area became a regression region some coagula became visible and these were left behind as the branch receded.

Rhythmic flow in the injected area was suppressed immediately or after a few minutes. Flow in the uninjected parts was resumed a few seconds after the injection at the same rate as before. It slowed down sometimes after 5 to 10 minutes, but it usually became more rapid for the next 10 to 20 minutes. There was also a weakening of pulsation, first limited to the site of injection. As the injected liquid spread throughout the whole culture, rhythmic pulsation was reduced everywhere.

There was a marked tendency for the reserve plasm to solate and for the viscosity of the plasmasol to be decreased. Evidence for the first conclusion consists in the porous appearance of the cultures which became noticeable after 5 minutes and spread rapidly over the whole colony. At the site of injection all of the plasmagel was attacked, so that after 30 minutes a regression region became established there, and after an hour the process of solation was complete. Most of the solated material collected in an uninjected pseudopod which became thick and swollen without showing any sign of forward flow. The most characteristic effect was the absence of large channels in the injected region. In normal colonies the streaming plasmasol collects in a few large veins which become firmly established after a short period of time. Following injection no sign of the formation of channels could be noticed. After 5 minutes all of the plasmasol was streaming through the network of small veins which are coextensive with the plasmagel of the pseudopod or the branch. There seemed to be even a tendency toward softening of the walls of the main branches. In many instances primordia of pseudopodial lobes began to form on the walls of the affected branches, a phenomenon which occurs rarely in normal cultures.

The size of all injected colonies seemed to be reduced, possibly by the loss of diffusible material. The severity of the final reactions depended on the size and condition of the injected colony as well as the dose. A large and healthy colony with a small dose of injection fluid returned to normal conditions after about an hour. In most of the colonies the effects lasted for two hours or more. Some of them became an unorganized mass of plasm without any perceptible motion.

Most of the observations are summarized in table 1.

DISCUSSION OF RESULTS

The interpretation of experimental results in biological material is always difficult owing to the extreme complexity of physiological functions both normal and experimental. The difficulty is even greater in our case for a number of reasons. The first difficulty, the high variability of the organism, was to a certain extent reduced by multiplying the number of experiments and concentrating only on those phenomena which showed up with regularity in the majority of the cases examined.

More serious is the difficulty of interpretation arising from the dubious state of purity of the enzyme preparations used. Papain and pectinase were very crude products. Furthermore, it would have been desirable to run control experiments with inactivated enzymes. Yet it was soon realized that it is very difficult to inactivate the enzymes in such a way that they could be used for the purpose of the present investigation. The only exception was pepsin which is quickly inactivated in a neutral medium. It turned out that the effects achieved with fresh papain solutions became less and less pronounced, as older solutions were injected, and were not present at all with solutions three hours after their preparation. This observation seemed to indicate that the specific results were due indeed to the presence of active enzyme.

Despite these uncertainties definite and consistent results have emerged from the experiments, and each enzyme produced a characteristic modification of structure and activity of the organism when injected into its protoplasm. From the observed modifications of structure and behavior inferences were made on the architecture of the underlying submicroscopic structure of the protoplasm of the slime mold, on the mechanism of solvation and gelation (which are the chief mechanisms of protoplasmic streaming), and on the chemical nature of the activation mechanism.

Review of experimental findings. A comparative study of table 1 reveals that each of the enzymes has indeed characteristic effects upon structure and activity of the mold, and that several of them have some effects in common. The enzymes used thus fall into three general classes — proteinases, collagenase, and polysaccharases plus ribonuclease. The common property of the proteinases is that all of them and they alone act upon the hyaline layer in such a way that hyaline protrusions are formed. Also they accelerate rhythmic flow and mobilize the reserve plasm, indicating that the viscosity of the plasmasol has been reduced. The differences are as follows: Papain is distinguished by the fact that it produces all signs of activation. Trypsin and pepsin cause an initial retardation of rhythmic flow which is followed by speeding up. Trypsin acts in very low concentrations probably because the pH of living protoplasm is close to the optimum pH of its activity. Trypsin prevents forward flow, the behavior of pepsin in this point being unclear. Pepsin has little effect upon the reserve plasm, possibly because it is so rapidly inactivated so that it does not reach the reserve plasm in an active state. Papain and pepsin result in more vigorous pulsation, while trypsin weakens it.

Collagenase resembles the other proteinases in that it acts upon the hyaline layer and on the reserve plasm. It differs from them by suppressing both forward and rhythmic flow. It also weakens pulsation. None of these enzymes initiates a regression region at the site of injection.

The third group of enzymes (pectinase, hyaluronidase and ribonuclease) is characterized by the fact that all of them act upon complexes containing carbohydrate or nucleic acid. These enzymes do not produce hyaline protrusions, they suppress forward flow and result in the formation of regression regions where they enter the protoplasm. Pectinase and hyaluronidase do not affect pulsation and accelerate rhythmic flow, while ribonuclease stops rhythmic flow and weakens pulsation. Hyaluronidase does not mobilize reserve plasm, while pectinase and ribonuclease do so.

The conclusions which were tentatively drawn from these findings are all based on two premises: (1) The commonly admitted assumption that frequently visible structures of an organism and its activity are manifestations of the molecular organization of its protoplasm at the submicroscopic level. One might expect that artificial modifications of the molecular make up of the protoplasm should be similarly reflected in its visible structure and activity. (2) The high substrate specificity of most enzymes by which their activity is limited to a certain substance or class of substances. As we have shown that every enzyme has its particular effect upon the structure and activity of the slime mold, the most acceptable conclusion would be that the particular substrate of each enzyme was present in the molecular framework of the protoplasm of this organism, particularly in those portions of the protoplasm where changes in structure and activity took place.

Composition of protoplasmic structures. The hyaline layer is affected by all proteinases and by these alone, which indicates that it is made up of simple, non-conjugated proteins. The plasmagel is attacked by the polysaccharases and ribonuclease (when these bring about its solation in the regression region) which indicates that it contains their specific substrates. The plasmasol is influenced by proteinases and polysaccharases (when they lower the viscosity and accelerate the rhythmic flow) probably through breakdown of high molecular weight compounds of protein and carbohydrate nature. The reserve plasm is mobilized by practically the same enzymes and hence is similar in its composition to the plasmasol. It may be regarded as plasmasol in a state of higher vicosity. Ribonuclease is set apart from the two polysaccharases by two important factors: It prevents the formation of channel walls, and coagula caused by it are not resorbed. From these findings one may infer that nucleoproteins have a large share in the make-up of channel walls, and that denaturation of the protoplasm induced by this enzyme is so severe that it becomes irreversible.

The mechanism of solation. As a result of our observations the following interpretation of solation is offered: It is assumed that the non-protein moieties upon which the polysaccharases and ribonuclease act are present in the plasmagel in the form of cross connections between protein chains. When solation occurs in the regression region these bonds are loosened. The proteins are apparently not attacked by proteinases as long as they are parts of the complexes, because these enzymes do not establish regression regions. As soon as the non-protein bridges have been broken down the protein chains become free and assume a globular shape. This follows from the fact that Lepeschkin ('50) was unable to observe flow birefringence in flowing plasmasol, and it is in agreement with the view of Goldacre and Lorch who maintain that the change from gel to sol in *Amoeba* is accompanied by a folding of protein chains. These folded proteins are probably identical with the prestructural elements postulated by Moore ('35), and by Moore and Winer ('41).

Now that the proteins are free they are open to the attack of proteolytic enzymes which lower the viscosity of the plasmasol. Polysaccharases also induce lowering of the viscosity, possibly by depolymerizing long chain molecules of their particular substrate. This conception of protoplasmic solation and its enzymatic character thus supplements the mechanisms proposed by De Bruyn ('47) in his recent discussion of protoplasmic streaming.

The mechanism of gelation. Far less can be said about protoplasmic gelation. It may be considered as the reverse process of solation. This means that those connections which solidify the protein chains to form a coherent network are reestablished. It is possible that gelation is catalyzed by the same enzymes as solation. The experimental results are not very conclusive on this point. The polysaccharases, ribonuclease and the proteinases in higher concentration prevent forward flow. The explanation is probably that the injection creates an excess of these enzymes in the protoplasm which prevent the reestablishment of those cross connections, resulting perhaps in the changes in consistency which are necessary for forward flow. This consideration points to the view that the amount of active enzyme present in a certain region of protoplasm determines the direction in which a chemical reaction is catalyzed, whether toward synthesis or toward degradation.

That the same enzyme is able to solate and to gelate protein solutions has been pointed out by Woodward ('48) who maintains that a trypsin-like or papain-like catheptic enzyme is a component of the normal mechanism of protein gelation considered as an enzymatic process. The earlier literature on the coagulating action of trypsin and papain is reviewed in the same paper. Woodward believes also that the action of the papainase is a slight denaturation of the protein chains which leads to their unfolding, which is again postulated by Goldacre and Lorch ('50) as prerequisite for gelation in *Amoeba.*

The nature of the activation mechanism. The hypothesis of the mechanism of protoplasmic gelation and solation in slime molds leads to a further hypothesis that the activation mechanism in these organisms depends on the activity of an enzyme system or systems. The experimental results which can be advanced in support of this view can be summarized as follows: Normal activity in slime molds can be intensified by injected papain; weakly streaming cultures can be stimulated to vigorous activity again by papain; vigorous activity is slowed down by trypsin and pepsin which points to the protein nature of the factor. The detailed effects of the enzymes in protoplasmic streaming are numerous, but they all point to a catalysis of protein degradation and protein coagulation. Protein degradation is required for the solation of the hyaline layer during the production of hyaline protrusions, for the decrease of viscosity of the plasmasol in order to speed up rhythmic flow, for the mobilization of reserve plasm and the breaking down of plasmagel at the regression area. Coagulation is necessary for the gelation of the hyaline layer after the production of protrusions and for the establishment of plasmagel.

The objection might be raised that inorganic ions such as those contained in Chambers' solution are also able to produce solation and gelation. This might be another instance of identical effects being caused by different agents. We do not know as yet whether protoplasmic gelation and solation are caused by ions or by enzymes alone, or by both agents and what the possible relationship of both agents might be. There is, however, one consideration which points to a predominant role of enzymes. Whatever the agent might be, it must be easily controlled and perhaps also reversible, and both requirements are satisfied by organic catalyses. Many of the papainases and cathepsins are SH-enzymes which are active only in the reduced state. The view that SH-enzymes play a decisive role in the reversible gel/sol changes provides for the possible regulation of these colloidal changes, as also pointed out by Woodward ('48). Barron ('51) has presented evidence for the existence of a mechanism for controlling the activity of SH-enzymes through the balance between cellular oxidation and glutathion production.

It is difficult to explain why papain and pepsin augment pulsation while trypsin, collagenase and ribonuclease weaken it. One possibility is that mobilization of reserve plasm empties the microscopic channels and thus facilitates the compression of pseudopods. This can, however, only be a partial explanation. Another possibility is that the mechanism underlying pulsation is activated by the first two enzymes and interfered with by the others. Another possibility is direct action of the enzymes upon the contractile elements, the energy production, or on the stimulating and coordinating mechanisms.

SUMMARY AND CONCLUSIONS

Certain features of the structure and activity of the slime mold *Physarum polycephalum* are described, particularly those which are modified by the procedure employed in the experiments described. Solutions were injected into the protoplasm of the mold. Potassium and sodium ions together enhance activity, while calcium ions depress it. To avoid these complications all inorganic ions were excluded from the dissolving medium and double distilled water was used as solvent for the enzymes.

The proteinases papain, trypsin and pepsin accelerate protoplasmic streaming and produce numerous protrusions at the hyaline layer. Collagenase also acts on the hyaline layer, but depresses streaming. The polysaccharases, pectinase and hyaluronidase, produce an increase in the rate of protoplasmic streaming, but depress the forward motion of the whole culture. Ribonuclease acts in a similar way, but in addition causes the breakdown or prevents the establishment of channel walls. The last three enzymes also promote the solation of plasmagel.

Tentative conclusions are drawn from the experimental findings. The hyaline layer seems to consist chiefly of nonconjugated proteins, while the plasmagel contains proteins in combination with other substances, such as polysaccharides and nucleic acid. These form cross connections between the protein chains. Solation and gelation may be interpreted as enzymatic processes involving the loosening and reestablishment of these connections. The activity of certain enzymes is responsible for vigorous activity of the plasmodium.

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LITERATURE CITED

Barron, Guzman, E. S. 1951 Thio groups of biological importance. Adv. Enzymol., *11:* 201–266.

Camp, W. G. 1936 A method of cultivating myxomycete plasmodia. Bull. Torrey Bot. Club, *63:* 205–210.

CAMP, W. G. 1937 The structure and activities of myxomycete plasmodia. Ibid., *64:* 307–335.

CHAMBERS, R. 1949 Micrurgical studies of protoplasm. Biol. Rev., *24:* 246–265.

DE BRUYN, P. H. 1947 Theories of amoeboid movement. Quart. Rev. Biol., *22:* 1–24.

GOLDACRE, R. J., AND I. J. LORCH 1950 Folding and unfolding of protein molecules in relation to cytoplasmic streaming, amoeboid movement and osmotic work. Nature, *166:* 497–500.

LEPESCHKIN, W. W. 1950 Über die Struktur und den molekularen Bau der lebenden Materie. Protoplasma, *39:* 222–244.

MAST, S. O. 1926 Structure and movement, locomotion and stimulation in Amoeba. J. Morph., *41:* 347–425.

MOORE, A. R. 1935 On the significance of cytoplasmic structures in plasmodium. J. Cell. and Comp. Physiol., *7:* 113–129.

WINER, B. J., AND A. R. MOORE 1941 Reactions of the plasmodium *Physarum polycephalum* to physico-chemical changes in the environment. Biodynamica, *3:* 323–345.

WOODWARD, A. A. 1948 Protoplasmic clotting in isolated muscle fibres. J. Cell. and Comp. Physiol., *31:* 359–394.

A COMPARISON OF THE LOCALIZATION OF ALKALINE GLYCEROPHOSPHATASE, AS DEMONSTRATED BY THE GOMORI-TAKAMATSU METHOD, IN FROZEN AND IN PARAFFIN SECTIONS [1, 2]

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SIXTEEN FIGURES

The critical study by Danielli in 1946 of the Gomori ('39, '41) and Takamatsu ('39) method for demonstrating alkaline glycerophosphatase revealed that a considerable proportion of enzyme activity is lost from tissues during the course of fixation and embedding. Danielli's experiments, however, led him to the conclusion that the intracellular localization of the precipitate formed by the enzymatic activity faithfully reflects the location of phosphatase. Since that time, a number of observations have led workers in the field to question the validity of Danielli's conclusion. First, although alkaline glycerophosphatase when demonstrated by the precipitation of calcium phosphate generally appears to be moderately concentrated in nuclei, alkaline phosphatase is generally absent from nuclei when demonstrated by the azo-dye techniques of Menten, Junge and Green ('44), or Manheimer and Seligman ('48) (Lorch, '47; Lison, '48; Yokoyama, Stowell and Mathews, '51; Gomori, '51). Nevertheless, Loveless and Danielli ('49), using a different azophosphate as substrate, obtained nuclear staining. Second,

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[1] This work was aided by funds from the Eugene Higgins Trust.

[2] This material was reported at the Third Annual Meeting of the Histochemical Society, April, 1952 (Herman and Deane, '52).

evidence of the diffusion of calcium phosphate from its site of formation to nuclei has been presented by Martin and Jacoby ('49), Gomori ('51), Leduc and Dempsey ('51), Novikoff ('51), and Moe ('52). Third, under most conditions, nuclei separated from other protoplasmic elements by ultracentrifugation display extremely little alkaline phosphatase activity (Yokoyama, Stowell and Tsuboi, '50; Novikoff, Podber and Ryan, '50; Novikoff, '52). It should be remembered, however, that Dounce ('50), using a different method of separation, recovered most of the alkaline phosphatase activity in the nuclear fraction.

Consideration of the observations listed above led us to the hypothesis that the processes of fixation, dehydration and embedding in paraffin somehow alter the nuclei so that they become stained by the calcium phosphate-cobaltous sulfide reagents in the alkaline phosphatase method. To test this hypothesis we have compared the staining of frozen sections with that of paraffin sections of the same organs. Blood smears and mesenteric spreads have been used to confirm the results with frozen sections.

MATERIAL AND METHODS

Liver, kidney, pancreas, mesentery and blood from laboratory rats (Sprague-Dawley strain) were used. The animals were killed by concussion. Small pieces of the organs (2 × 5 mm) were removed. Pieces of mesentery were spread on cover glasses, and smears were made of blood removed from the tail. Each tissue or organ was studied from at least two and generally 5 animals.

Paraffin sections. Blocks of tissue were immersed directly in 80% alcohol chilled to 4°C. and fixed for 24 hours. They were dehydrated through 95% alcohol (24 hours) and absolute alcohol (3 hours), cleared in chloroform (overnight), infiltrated with paraffin at 48°C. ($1\frac{1}{2}$ hours) and embedded. Sections were cut at 5 μ, mounted on slides coated with albumen and dried for two days at room temperature.

Frozen sections. Similar blocks of tissue were placed immediately in a copper cup chilled to — 30°C. in a deep freeze unit. As soon as the blocks were completely hardened, they were transferred to a covered glass container to prevent severe dehydration. Sections were cut at 5 µ in a modified Linderstrøm-Lang cryostat (Coons, Leduc and Kaplan, '51). They were mounted on uncoated slides, dried thoroughly at room temperature and stored at 4°C. Preliminary experiments indicated that there was no loss of enzymatic activity with storage of the sections at 4°C. for as long as a year. In all of the reported experiments, however, the slides were used within one week after cutting.

Mesenteric spreads. Pieces of mesentery were spread on cover glasses and immersed before drying in chilled 80% alcohol for 30 minutes, rinsed in distilled water, and then stained with the other sections.

Blood smears. Blood smears on cover glasses were thoroughly dried at room temperature, fixed in chilled 80% alcohol for 30 minutes and dried. They were used immediately or stored in the refrigerator for as long as a week, with no detectable difference in stainability.

Staining. In general the frozen sections were fixed just prior to staining in chilled 80% alcohol for 30 minutes and then rinsed in distilled water. However, some of the frozen sections were stained without first being fixed. The paraffin sections were deparaffinized and run down to distilled water. No celloidin coating was used.

With the exception of certain controls to be mentioned below, all sections were incubated at 37°C. for periods ranging from 15 minutes to 24 hours (15, 30, and 45 minutes; 1, 3, 5 and 24 hours) in the following standard medium, pH 9.5:

2% α-β sodium glycerophosphate	20 cm³
2% sodium barbital	20 cm³
2% calcium chloride, anhydrous	10 cm³
distilled water	50 cm³
magnesium chloride solution [3]	1.9 cm³

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[3] The magnesium chloride solution was made as follows: 1.65 g $MgCl_2 \cdot 6H_2O$ and 20 cm³ of 2% sodium barbital, made up to 100 cm³ with distilled water (Morse and Greep, '47).

The final concentration of calcium chloride in this solution is 0.02M, and that of magnesium chloride 0.0015M.

After incubation, the slides were treated with two changes of 1% calcium chloride for 10 minutes, 2% cobaltous nitrate for two minutes, several changes of distilled water, and 1% yellow ammonium sulfide for two minutes. They were then thoroughly washed with running tap water, dehydrated, cleared and mounted in Permount.

Modifications and controls. In order to determine at what step in the preparation of paraffin sections the staining properties of the nuclei are changed, the following experiment was set up. Several series of frozen sections and of mesenteric spreads were treated as follows: 1 slide was fixed in chilled 80% alcohol for 30 minutes only; a 2nd was fixed in 80% alcohol for 24 hours; a 3rd was treated with 80% alcohol for 24 hours, and 95% alcohol for 24 hours; a 4th was treated with 80% alcohol for 24 hours, 95% alcohol for 24 hours, and absolute alcohol for 3 hours; a 5th was treated with 80% alcohol for 24 hours, 95% alcohol for 24 hours, absolute alcohol for 3 hours, and chloroform overnight; a 6th was treated with 80% alcohol for 24 hours, 95% alcohol for 24 hours, absolute alcohol for 3 hours, chloroform overnight, and paraffin at 48°C. for 1½ hours. The tissues that had been paraffinized were treated with xylene and all slides were hydrated. They were stained in the standard medium given above.

Since it has been suggested that diffusion of phosphatase or of calcium phosphate from the site of formation to other sites may occur, we compared sections of liver and kidney incubated in media to which various salts had been added with other sections incubated in the standard medium. (1) The calcium chloride concentration was raised from 0.02M to 0.06M and 0.1M (Gomori, '51). (2) Sodium sulfate was added to final concentrations of 0.06M, 0.1M, 0.5M, and 1.4M (Koelle, '51). (3) Sodium chloride was added in final concentration of 2M (Seligman and Manheimer, '49).

To test the activating effect of magnesium chloride (Maengwyn-Davies and Friedenwald, '50), a series of slides was incubated in the medium with magnesium omitted.

Finally, three types of controls were set up:

1. In order to test whether or not nuclei attract calcium phosphate, two experiments were performed: (a) The standard incubating medium was saturated with dibasic calcium phosphate (Maengwyn-Davies and Friedenwald, '50); (b) sections were incubated in the buffer solution saturated with dibasic calcium phosphate.
2. To rule out any effect of albumen on the phosphatase reaction, we used uncoated as well as albumen-coated slides for both paraffin and frozen sections.
3. The usual controls omitting glycerophosphate from the incubating medium were made.

RESULTS

Frozen sections, briefly fixed in 80% alcohol, generally show earlier staining than do paraffin sections from the same organ. Thus bile capillaries are blackened in frozen sections of liver within two hours, whereas paraffin sections require 4 or 5 hours and even then are only irregularly stained (figs. 9, 10). Similarly in frozen sections of pancreas, all of the peri-acinar and islet capillaries are deep black in three hours, while in paraffin sections only a few vessels display significant staining at that time (figs. 4, 5).

Tissue constituents in frozen sections which blacken within 30 minutes include the brush borders of kidney tubules and the adventitial coat of arteries and arterioles (fig. 13), and the granules of neutrophilic leukocytes observed in blood smears and within blood vessels in liver and pancreas. After three hours, the stroma of the bile ducts, hepatic arteries and the bile capillaries, the alpha cells of the pancreatic islets and the stroma of the pancreatic ducts (fig. 4) are well stained. Also the cytoplasmic granules of tissue eosinophils in the mesentery (fig. 2) are blackened. We have been unable to determine whether eosinophils in the blood stream also stain, since eosinophils and neutrophils are difficult to distinguish. This problem does not occur in the mesenteric spreads, since neutrophils are very sparse or entirely absent in the stroma of the normal mesentery. It is worthy of note that the mast cell granules are virtually unstained with short periods of incubation but become intensely blackened after 24 hours (fig. 3).

Striking differences are shown in the period of incubation necessary to reveal pronounced alkaline glycerophosphatase activity in the rat organs and tissues which were studied. Thus a 30 minute incubation period produces significant deposits in kidney, whereas a three hour incubation period is necessary for blood, pancreas and mesentery, and a 5 hour period for liver. These differences may provide rough quantitative estimates of the amount of enzymatic activity in these sites.

It is interesting that capillaries in various organs exhibit different degrees of activity. Thus, while the walls of the capillaries in the pancreas (fig. 4) and mesentery stain intensely within three hours, both the capillaries of the kidney (fig. 13) and the sinusoids of the liver (fig. 8) fail to stain even with incubation up to 24 hours.

In frozen sections or in fresh smears and spreads, which have been fixed for a brief time, the nuclei fail to stain with short periods of incubation (figs. 2, 4, 8, 9, 13), whereas the nuclei are among the first structures to stain in paraffin sections (figs. 5, 10, 14). In the paraffin sections, structures other than nuclei, such as the pancreatic capillaries and the bile capillaries, are much more faintly stained than in frozen sections, even after long incubation. It is suggested, therefore, that in the paraffin sections the nuclei have adsorbed either phosphatase or a salt produced after hydrolysis of the substrate. Prolonging the incubation period of frozen sections to 24 hours results in increasing nuclear staining (figs. 3, 6, 11), although it never becomes so great as in the paraffin sections (figs. 7, 12).

Frozen sections which have not been fixed at all stain even more intensely than frozen sections fixed briefly in alcohol (figs. 8, 9, 13, 15). The localization of precipitate, however, is less definite, and in the kidney at least, nuclear staining becomes evident early (fig. 15).

Since it has been suggested that diffusion of calcium phosphate from the sites of formation to other locations may occur (see introduction), the effect of increasing salt concentration in the incubating medium was investigated. In agreement with Gomori ('51), we find that increasing the calcium chloride concentration from 0.02M to 0.06M or 0.1M reduces the amount of nuclear and cytoplasmic staining in the vicinity of intensely active structures (compare figs. 15 and 16). The overall activity appears to be slightly reduced, but this may be an optical illusion resulting from the limited localization of the precipitate. On the other hand, with both sodium sulfate and sodium chloride, which have been reported to diminish enzyme migration, marked inhibition of activity occurs without any improvement of localization (sodium sulfate, 0.06M, 0.1M, 0.5M, and 1.4M — Koelle, '51; sodium chloride, 2M — Seligman and Manheimer, '49).

To determine at what step in the preparation of paraffin sections the nuclei are altered so as to become stainable, frozen sections and mesenteric spreads were run up variously to higher alcohols, chloroform and paraffin (p. 204). The preparations which were treated with alcohols, even for prolonged periods, show little or no nuclear staining. In contrast, those which were run up to chloroform or to paraffin show moderate coloration of the nuclei, intermediate between that seen in briefly fixed frozen sections and sections from paraffin blocks.

Our standard incubation medium contains magnesium, which is regarded as an activator of alkaline phosphatase (Bodansky, '36; Maengwyn-Davies and Friedenwald, '50). To determine if the presence of magnesium in the medium might account for some nuclear activity we incubated paraffin and frozen sections of the same tissues in two media, one containing magnesium chloride and the other lacking this salt. In our experience the overall activity is increased by the presence of magnesium, but no differences emerge in the relative concentration of precipitate in various sites.

Other controls gave only negative results. Thus sections incubated in a medium lacking glycerophosphate showed no colored precipitate. Sections incubated in a buffer solution saturated with dibasic calcium phosphate also displayed no precipitate. Saturating the standard medium with dibasic calcium phosphate (Maengwyn-Davies and Friedenwald, '50) showed no alteration in the amount of precipitate. A comparison of sections mounted on uncoated slides and slides coated with albumen also revealed no differences.

DISCUSSION

The use of paraffin-embedded tissues appears to be responsible for an ''artefactual'' staining of nuclei in the Gomori-Takamatsu method for alkaline glycerophosphatase. Sections from the same organs cut unfixed in a cryostat and then fixed briefly in 80% alcohol fail to show any nuclear staining unless the period of incubation is extended beyond 5 hours. Similarly, in the past, blood smears have been reported to contain little or no nuclear alkaline phosphatase (Rheingold and Wislocki, '48), although paraffin sections of blood cells reveal considerable nuclear activity.

Several explanations for the nuclear staining suggest themselves. (1) It is possible that the enzyme itself diffuses from sites of high activity to the nuclei (Feigin, Wolf and Kabat, '50). This appears unlikely since Leduc and Dempsey ('51) found that immersion of inactivated sections in a solution of alkaline phosphatase failed to show nuclear adsorption at alkaline pH, although such adsorption did occur at acid pH. Moog ('51) adduced additional evidence that the enzyme itself does not migrate. Moreover, our attempts to prevent protein migration with sodium sulfate or sodium chloride merely served to inhibit enzyme activity, without affecting location of the precipitate. (2) The more likely possibility is that the calcium phosphate itself migrates from the site of its formation to the nuclei, since increased concentration of calcium in the incubating medium suppresses nuclear staining. This explanation is supported by Gomori's experiments ('51) and is confirmed by the present investigation. Feigin and Wolf ('52) have suggested that the cobaltous sulfide may be the substance which migrates. The lack of nuclear staining in frozen sections, however, makes the migration of cobaltous sulfide a doubtful cause of the staining in paraffin sections. (3) A third possibility remains, namely, that treatment with solvents and heat somehow unmasks enzyme activity which is not detectable in fresh or briefly fixed sections. This possibility appears remote, since such measures are not beneficial to protein enzymes.

Possibly related to the secondary nuclear staining is the belated staining of mast cell granules, which are also highly acidic. In mesenteric spreads incubated for a short period, the only granulocytes to stain are the tissue eosinophils. When the incubation period is extended to 24 hours, however, the eosinophils have lost most of their activity whereas the mast cell granules have become intensely stained. This time factor probably explains the difference between our results and those of Wislocki and Fawcett ('51), since they based their conclusions principally on results obtained with long incubation times (personal communication). Riley and Drennan ('49) also disagree with the earlier reports that mast cell granules are intensely reactive for alkaline phosphatase. In sections of skin, they found only the smaller and apparently younger mast cells to be reactive. In our opinion, this reaction, rather than being due to the youth of these cells as postulated by Riley and Drennan, may have been due to proximity of the mast cells to highly active blood vessels, from which secondary transfer of calcium phosphate may have occurred.

According to Goetsch, Reynolds and Bunting ('52), allowing sections to remain embedded in paraffin during the staining procedure also prevented nuclear staining in both the alkaline and acid phosphatase methods, although ample staining occurred in the customary non-nuclear sites.

In contrast to the present results, several investigators have described marked nuclear activity in thin tissue cultures which were stained in toto (e.g. Chèvremont and Firket, '49; Biesele and Wilson, '51). In both of these experiments, the incubation time was at least 18 hours, a period which might permit migration of the precipitate to nuclear structures. Moreover, Biesele and Wilson obtained only weak nuclear staining when glycerophosphate was employed, although muscle adenylic acid produced marked staining (Wachstein and Meisel, '52).

Chèvremont and Firket used glycerophosphate only and obtained striking deposition on nuclei and mitotic chromosomes. One problem in the interpretation of the results with tissue cultures resides in the fact that the explant from which the flattened cells migrate is large and its presence may cause the production of large quantities of calcium phosphate which might transfer to the nuclei of neighboring cells. Our own observations on mesenteric spreads make this interpretation seem likely, since, with short incubation, the nuclei of mesothelial cells appear entirely unstained in the "windows" between blood vessels, whereas those overlying the intensely active arterioles and capillaries are positive after only three hours' incubation.

The present experiment was conducted entirely with glycerophosphate. There is considerable evidence that other substrates produce conspicuous nuclear staining even in smears or frozen sections. This is particularly true when muscle adenylic acid (Wachstein and Meisel, '52) or nucleic acids (Friedenwald and Crowell, '49) are employed. Phosphatases for adenine phosphates and nucleic acids might be expected to occur where their substrates are normally concentrated. Further study on the specificity of individual phosphatases is needed.

In conclusion, it would seem wise from the results reported here to employ briefly fixed frozen sections and short incubation periods (Moog, '51; Moe, '52) if one wishes to obtain a true localization of alkaline glycerophosphatase activity. Ordinary paraffin embedding and prolonged incubation favor migration and adsorption of the products of hydrolysis, markedly changing the pattern of distribution of the reaction. One cannot be entirely confident, of course, that even the measures recommended here are sufficient to prevent artefacts. Nevertheless, it is gratifying to note that our observations by histochemical methods are more closely in agreement with results of biochemical studies (Yokoyama et al., '50; Novikoff, '52) than results heretofore reported.

SUMMARY AND CONCLUSIONS

Frozen and paraffin sections of liver, kidney and pancreas from rats are compared for alkaline glycerophosphatase activity by the Gomori-Takamatsu method.

In general, frozen sections stain more quickly than paraffin sections and display the precipitate in fewer locations, suggesting a lack of diffusion.

Parallel paraffin sections show prominent nuclear staining, but less staining in sites blackened in frozen sections.

In mesenteric spreads the granules of tissue eosinophils stain early, whereas after longer incubation they cease to blacken while the mast cell granules become progressively more deeply stained.

The results are discussed in the light of theories of migration of various substances during the process of the demonstration of the enzyme. It is concluded that they are most consistent with migration and adsorption of calcium phosphate on structures of high acidity, such as nuclei and mast cell granules.

The results obtained indicate the advantage of using frozen sections and short incubation periods if one wishes to obtain a true localization of alkaline glycerophosphatase activity.

ADDENDUM

Since this manuscript was prepared, additional reports have appeared of experiments designed to test the validity of localization in the alkaline glycerophosphatase method. In agreement with the present interpretation that diffusion of the calcium phosphate may play an important role in the location of the final product, Fredricsson ('52) observed that by employing an incubation mixture containing 40% acetone, the nuclei failed to stain even following prolonged incubation. Such a concentration of acetone was demonstrated to reduce the solubility of calcium phosphate but to affect the activity of the enzyme only slightly.

On the other hand, Ruyter ('52) has concluded that the cobaltous-sulfide method fails to reveal calcium phosphate when concentration of the latter exceeds a certain threshold value. Parallel preparations of intestine were incubated in glycerophosphate, and the calcium phosphate of one was then converted to cobaltous sulfide while that of the second was converted to reduced silver (von Kossa's method). When the incubation period was long, the first preparation gave a negative reaction in the striated border, whereas the silvered preparation showed an intense coloration. Subsequent exposure of the first preparation to silver nitrate was reported to blacken the border intensely, suggesting that cobaltous sulfide had failed to replace the dense concentration of calcium phosphate.

LITERATURE CITED

Biesele, J. J., and A. Y. Wilson 1951 Alkaline phosphatase substrate specificities in cultured normal and malignant cells of mouse, rat and fowl. Cancer Res., *11:* 174–179.

Bodansky, O. 1936 The effect of α-amino acids and magnesium on the activity of kidney and intestinal phosphatases. J. Biol. Chem., *115:* 101–110.

Chèvremont, M., and H. Firket 1949 Phosphatase alcaline dans les cellules cultivées in vitro; ses rapports avec la croissance. Arch. Biol., *60:* 441–472.

Coons, A. H., E. H. Leduc and M. H. Kaplan 1951 Localization of antigen in tissue cells. VI. The fate of injected foreign proteins in the mouse. J. Exp. Med., *93:* 173–188.

Danielli, J. F. 1946 A critical study of techniques for determining the cytological position of alkaline phosphatase. J. Exp. Biol., *22:* 110–117.

Dounce, A. L. 1950 Enzyme systems of isolated cell nuclei. Ann. N. Y. Acad. Sci., *50* (Art. 8): 982–999.

Feigin, I., and A. Wolf 1952 Nuclear staining in the Gomori method for alkaline phosphatase. J. Nat. Cancer Inst., *13:* 250 (Abstract).

FEIGIN, I., A. WOLF AND E. A. KABAT 1950 Histochemical studies on tissue enzymes. VI. A difficulty in the histochemical localization of alkaline phosphatase in nuclei. Am. J. Path., *26:* 647–659.

FREDRICSSON, B. 1952 A modification of the histochemical method for demonstration of alkaline phosphatase in which the nonspecific reactions and the diffusion phenomena are reduced. Anat. Anz., *99:* 97–106.

FRIEDENWALD, J. S., AND J. E. CROWELL 1949 Histochemical studies on nucleic acid phosphatase. Bull. Johns Hopkins Hosp., *84:* 568–582.

GOETSCH, J. B., P. M. REYNOLDS AND H. BUNTING 1952 Modification of Gomori method for alkaline and acid phosphatase avoiding artefact staining of nucleus. Proc. Soc. Exp. Biol. Med., *80:* 71–75.

GOMORI, G. 1939 Microtechnical demonstration of phosphatase in tissue sections. Proc. Soc. Exp. Biol. Med., *42:* 23–26.

———— 1941 The distribution of phosphatase in normal organs and tissues. J. Cell. and Comp. Physiol., *17:* 71–84.

———— 1951 Alkaline phosphatase of cell nuclei. J. Lab. and Clin. Med., *37:* 526–531.

HERMAN, E., AND H. W. DEANE 1952 Comparison of alkaline phosphatase activity in frozen and paraffin sections. J. Nat. Cancer Inst., *13:* 248–249 (Abstract).

KOELLE, G. B. 1951 Elimination of enzyme diffusion artefacts in histochemical localization of cholinesterases. Fed. Proc., *10:* 315.

LEDUC, E. H., AND E. W. DEMPSEY 1951 Activation and diffusion as factors influencing the reliability of the histochemical method for alkaline phosphatase. J. Anat., *85:* 305–315.

LISON, L. 1948 Le recherche histochimique des phosphatases. Étude critique. Bull. d'Histol. Appli., *25:* 23–41.

LORCH, J. 1947 Note on the cytological localization of alkaline phosphatase. Quart. J. Micro. Sci., *88:* 159–161.

LOVELESS, A., AND J. F. DANIELLI 1949 A dye phosphate for the histo- and cytochemical demonstration of alkaline phosphatase, with some observations on the differential behavior of nuclear and extranuclear enzymes. Quart. J. Micro. Sci., *90:* 57–66.

MAENGWYN-DAVIES, G. D., AND J. S. FRIEDENWALD 1950 Histochemical studies of alkaline phosphatases in the tissues of the rat using frozen sections. J. Cell. and Comp. Physiol., *36:* 421–459.

MANHEIMER, L. H., AND A. M. SELIGMAN 1948 Improvement in the method for the histochemical demonstration of alkaline phosphatase and its use in a study of normal and neoplastic tissues. J. Nat. Cancer Inst., *9:* 181–199.

MARTIN, B. F., AND F. JACOBY 1949 Diffusion phenomenon complicating the histochemical reaction for alkaline phosphatase. J. Anat., *83:* 351–363.

MENTEN, M. L., J. JUNGE AND M. H. GREEN 1944 A coupling histochemical azo dye test for alkaline phosphatase in the kidney. J. Biol. Chem., *153:* 471–477.

MOE, H. 1952 On the histochemical demonstration of alkaline glycerophosphatase and its dependence on the incubation time. Studies on the duodenum and jejunum of the cat and mouse. Anat. Rec., *112:* 217–236.

MOOG, F. 1951 Nondiffusibility of alkaline phosphatase in fixed tissue. Science, *114:* 109.

MORSE, A., AND R. O. GREEP 1947 Alkaline glycerophosphatase in the developing teeth of the rat. Anat. Rec., *99:* 379–396.

NOVIKOFF, A. B. 1951 The validity of histochemical phosphatase methods on the intracellular level. Science, *113:* 320–325.

——— 1952 Histochemical demonstration of nuclear enzymes. Exp. Cell. Research, Supplement *2:* 123–140.

NOVIKOFF, A. B., E. PODBER AND J. RYAN 1950 Intracellular distribution of phosphatase activity in rat liver. Fed. Proc., *9:* 210.

RHEINGOLD, J. J., AND G. B. WISLOCKI 1948 Histochemical methods applied to hematology. J. Hematol., *3:* 641–655.

RILEY, J. F., AND J. M. DRENNAN 1949 The presence and significance of alkaline phosphatase in the cytoplasm of mast cells. J. Path. and Bact., *61:* 245–251.

RUYTER, J. H. C. 1952 Histochemically detectable alkaline phosphatase in the cuticular border of the epithelium of the small intestine. Acta Anat., *16:* 209–220.

SELIGMAN, A. M., AND L. H. MANHEIMER 1949 A new method for the histochemical demonstration of acid phosphatase. J. Nat. Cancer Inst., *9:* 427–434.

TAKAMATSU, H. 1939 Histologische und biochemische Studien über die Phosphatase. Histochemische Untersuchungsmethodik der Phosphatase und deren Verteilung in verschiedenen Organen und Geweben. Trans. Soc. Path. Jap., *29:* 492–498.

WACHSTEIN, M., AND E. MEISEL 1952 Histochemical demonstration of 5-nucleotidase activity in cell nuclei. Science, *115:* 652–653.

WISLOCKI, G. B., AND D. W. FAWCETT 1951 Some histochemical properties of mast cells and tissue eosinophils in stained spreads of normal rat mesentery. J. Nat. Cancer Inst., *12:* 258.

YOKOYAMA, H. O., R. E. STOWELL AND R. M. MATHEWS 1951 Evaluation of histochemical alkaline phosphatase technics. Anat. Rec., *109:* 139–160.

YOKOYAMA, H. O., R. E. STOWELL AND K. K. TSUBOI 1950 Evaluation of the histochemical phosphatase techniques. J. Nat. Cancer Inst., *10:* 1367.

PLATES

PLATE 1

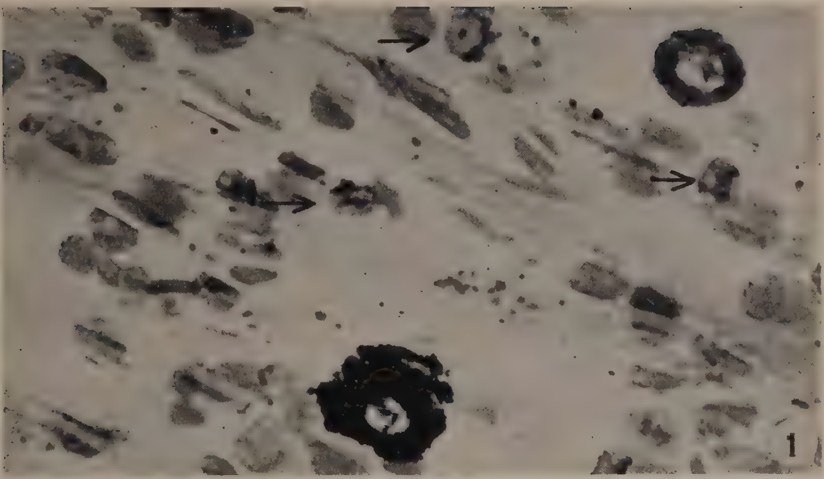
EXPLANATION OF FIGURES

Mesenteric spreads

1 Spread of mesentery, stained with Wright's mixture and photographed with a blue-green filter (Wratten no. 68). Two darkly stained mast cells and a number of eosinophils (arrows) are present in this field. The streaks represent the negative images of connective tissue fibers. × 600.

2 Spread of mesentery, fixed for 30 minutes in 80% alcohol; alkaline glycerophosphate medium three hours. Photographed with green and yellow filters (Wratten B_2, no. 58; G, no. 15). Four eosinophils are intensely stained, whereas the mast cell (arrow) and cell nuclei are barely discernible. × 600.

3 Spread of mesentery, fixed for 30 minutes in 80% alcohol; alkaline glycerophosphate medium for 24 hours. Photographed and printed exactly as figure 2. The mast cell granules have become intensely stained and the nuclei moderately darkened, whereas the cytoplasm of the eosinophil (arrow) has lost its stain. × 600.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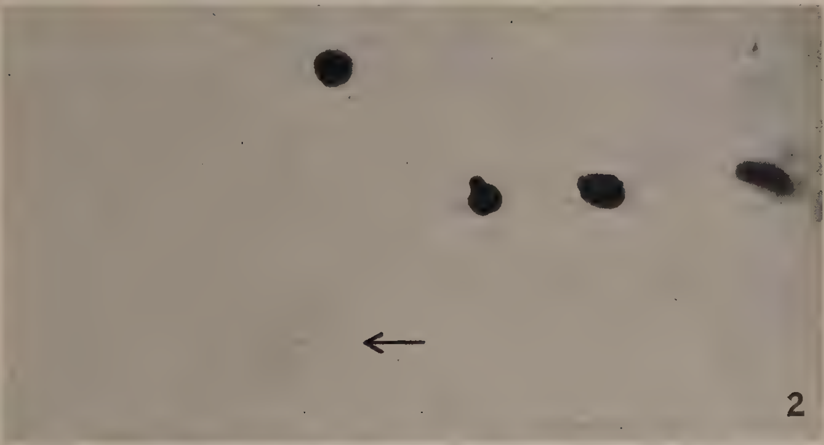
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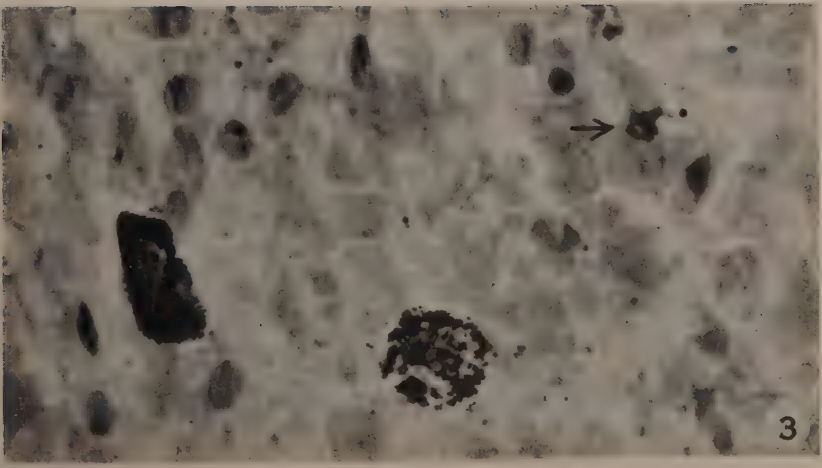
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PLATE 2

EXPLANATION OF FIGURES

All photomicrographs on plates 2, 3 and 4 were made and printed identically. Green and yellow filters (Wratten B_2, no. 58; G, no. 15). × 250.

Pancreas

In each photomicrograph, a duct lies in the upper portion of the field, an islet of Langerhans in the lower portion.

Sections 4 and 5 were incubated in glycerophosphate medium for three hours.

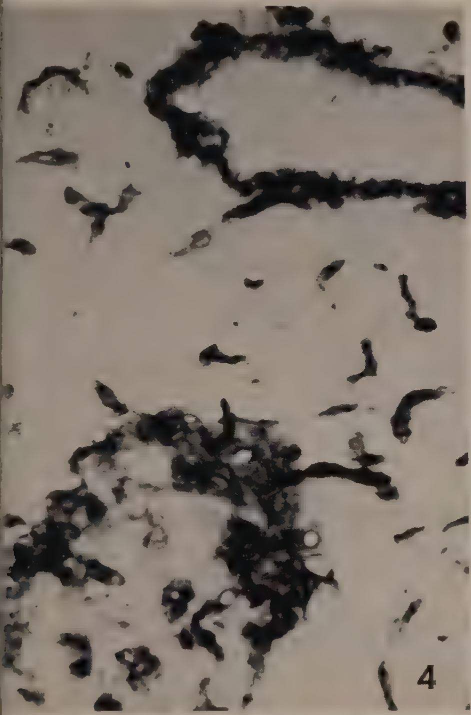
4 Pancreas, frozen section fixed for 30 minutes in 80% alcohol. The stroma of the pancreatic duct, the walls of the capillaries, and the alpha cells in the islet are stained; the nuclei are unstained.

5 Pancreas, paraffin section. The stroma of the duct, occasional capillaries, and the alpha cells are stained. Nuclei are slightly stained, more so in the alpha cells.

Sections 6 and 7 were incubated in glycerophosphate medium for 24 hours.

6 Pancreas, frozen section fixed for 30 minutes in 80% alcohol. The overall staining appears to be less intense than in figure 4, but the nuclei have become visible.

7 Pancreas, paraffin section; all nuclei are now intensely stained, whereas the capillary walls are less active than those in figures 4 and 6.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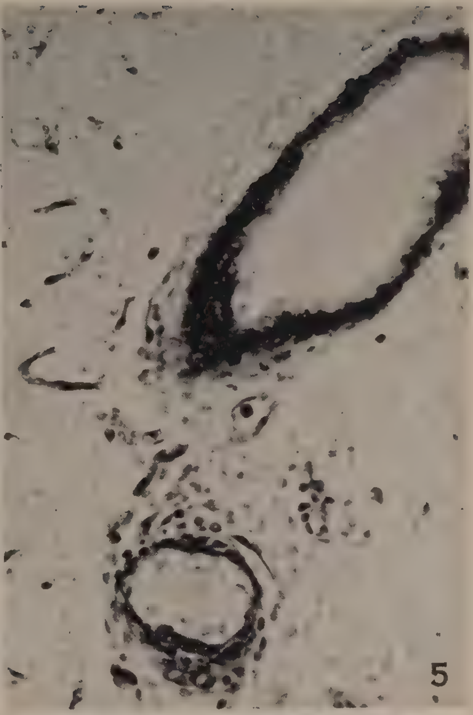
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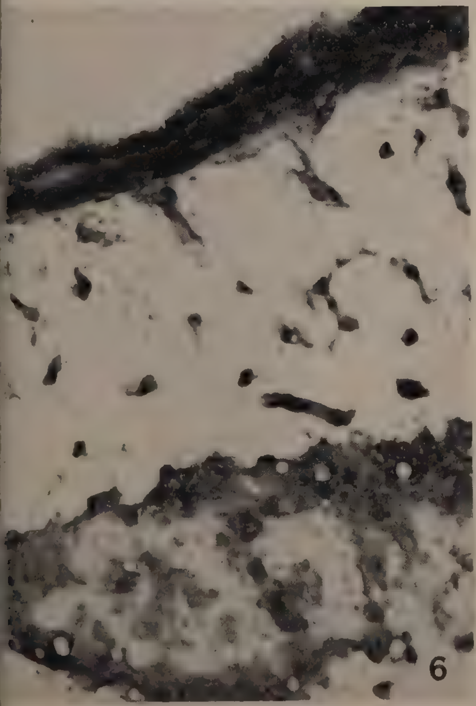
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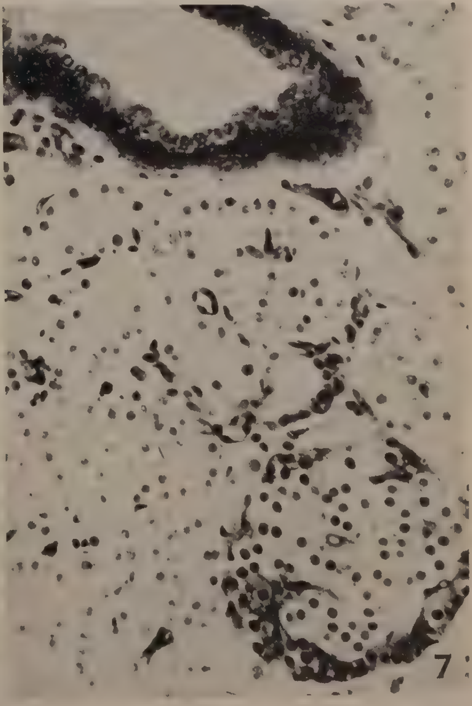
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PLATE 3

EXPLANATION OF FIGURES

Liver

Sections 8, 9 and 10 were incubated in glycerophosphate medium for 5 hours.

8 Liver, frozen section, unfixed. The wall of the bile duct and the bile capillaries are intensely stained, as are neutrophils (arrow) in the sinusoids.

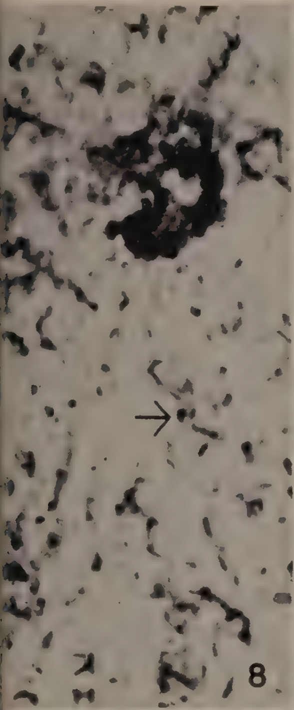
9 Liver, frozen section, fixed for 30 minutes in 80% alcohol. The distribution of staining is the same as in figure 8, but less precipitate has formed. Arrow, neutrophil.

10 Liver, paraffin section. The wall of the bile duct and neutrophils (arrow) are stained, but the bile capillaries are not. Nuclei are weakly stained.

Sections 11 and 12 were incubated in glycerophosphate medium for 24 hours.

11 Liver, frozen section, fixed for 30 minutes in 80% alcohol. The overall intensity is slightly greater than in figure 9, but the nuclei are now faintly stained.

12 Liver, paraffin section. The nuclei are more intense than in figure 10; some bile capillaries (arrow) are faintly stained.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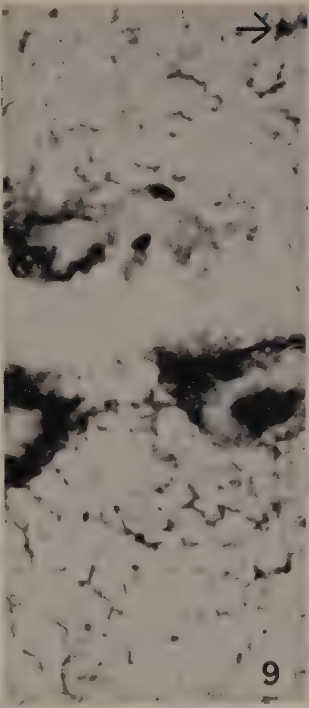
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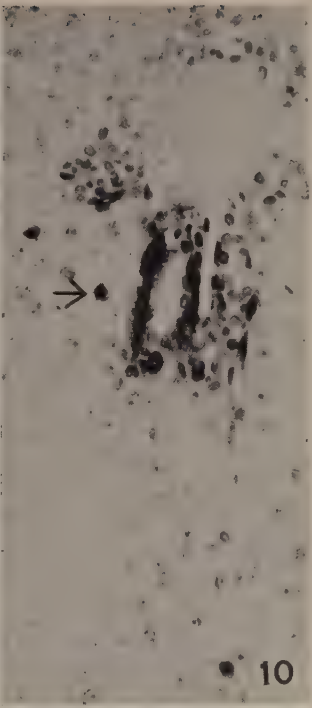
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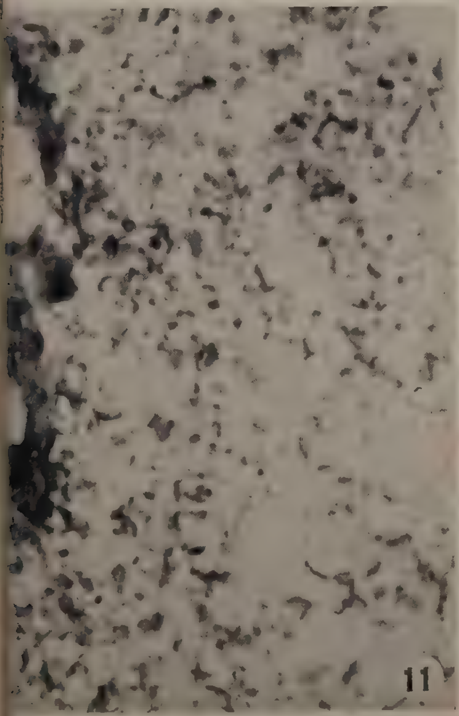
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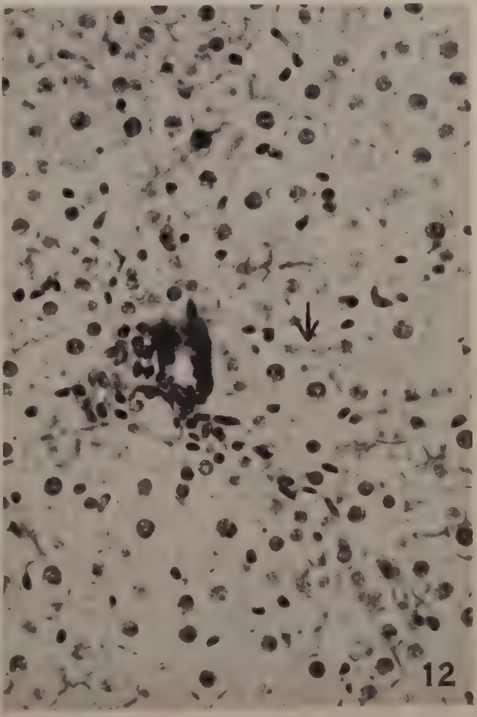
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PLATE 4

EXPLANATION OF FIGURES

Kidney

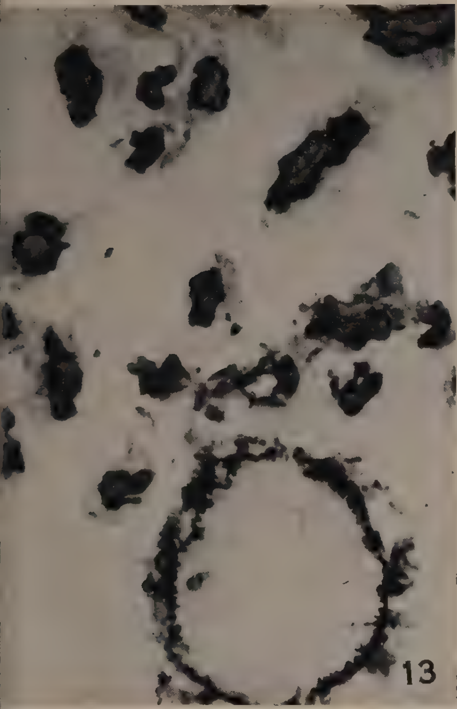
In each photomicrograph, kidney tubules above and a small artery below. All sections incubated for 30 minutes.

13 Kidney, frozen section, fixed for 30 minutes in 80% alcohol, incubated in standard medium. The brush borders of the proximal tubules and the externa of the artery are stained.

14 Kidney, paraffin section, incubated in standard medium. In addition to the brush borders and the externa of the artery, the cytoplasm and the nuclei of the tubular cells are faintly stained.

15 Kidney, frozen section, unfixed, incubated in standard medium. Heavier precipitate than in figure 13, with the nuclei faintly stained.

16 Kidney, frozen section, unfixed, incubated in medium containing 0.1M $CaCl_2$. The precipitate appears to be essentially as heavy as in figure 15, but the nuclei are not stained.



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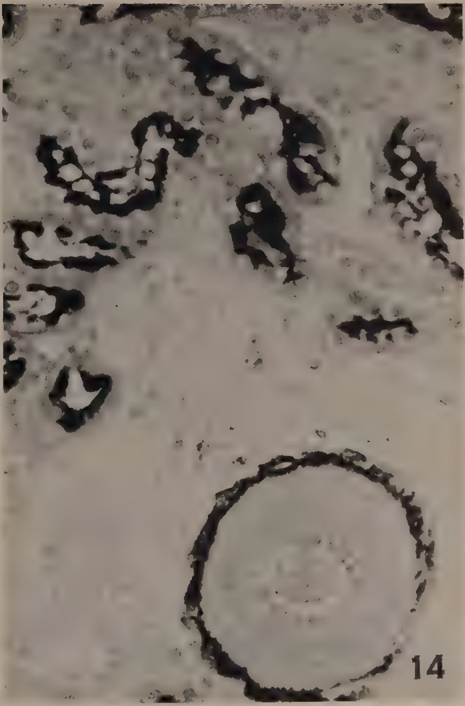

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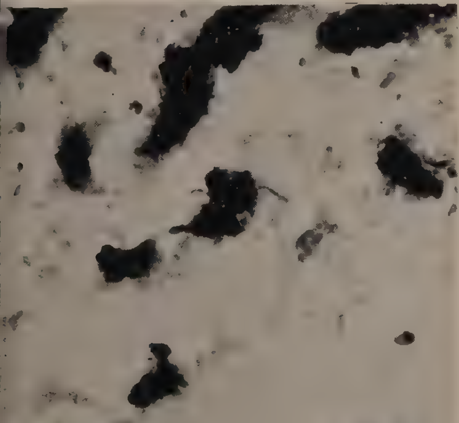

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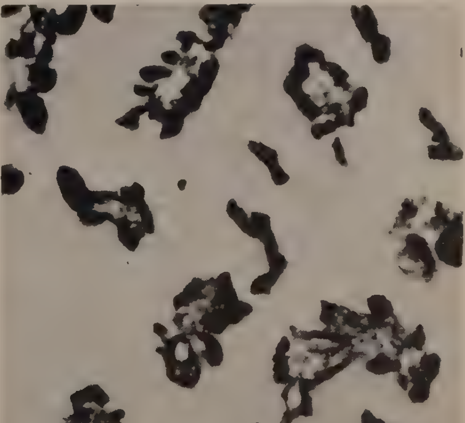

16

RATES OF EXCHANGE OF PHOSPHATE IONS IN HUMAN, RABBIT, AND CHICKEN BLOOD[1]

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TWO FIGURES

Considerable success has been achieved in quantitative studies of the exchange of cations between the plasma and erythrocytes of various species *in vitro* (Sheppard and Martin, '50; Sheppard, Martin and Beyl, '51; Raker, Taylor, Weller and Hastings, '50). Although the kinetics involved in cation exchange are by no means simple, a study of the exchange of orthophosphate anions in such systems presents additional difficulties. For example, exchange of potassium ions between plasma and blood cells lends itself very well to kinetic analysis based upon a two-compartment system (Sheppard and Martin, '50). However, when phosphate ions enter blood cells, they quickly become bound with organic moieties to form phosphate esters which occur only intracellularly. Thus in addition to two major compartments, the cells and the plasma, there may be in the case of phosphate ions an unknown number of minor "compartments" consisting of the various phosphate esters in the cells.

Kinetic analysis of the exchange of potassium ions between blood cells and plasma is further simplified by the fact that it is possible to maintain this system *in vitro* in a biologically steady state, as shown by relatively constant cell and plasma potassium contents for several hours. In the case of phosphate ions it is extremely difficult to maintain a state of dynamic equilibrium in whole blood since the rate at which phosphate ions exchange is extraordinarily sensitive to small changes in blood pH (Rapoport and Guest, '39). When the blood pH falls below 7.3, phosphorolysis occurs in the cells and the rate at which phosphate ions leave the cells is greater than the rate at which they enter the cells. At a blood pH of 7.4 or higher the reverse occurs: rephosphorylation is favored and the rate at which phosphate ions enter the cells is greater than the rate at which they leave the cells.

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[1] This work was carried out under contract No. AT-(40-1)-263 with the Medical Branch, Division of Biology and Medicine of the U. S. Atomic Energy Commission. A preliminary report has been published (Federation Proc., *11:* 57, 1952).

Maintenance of a constant pH when whole blood is incubated *in vitro* is not easy. If human blood is incubated with 5% CO_2 and 95% O_2 for a period of three hours the pH typically falls and the concentration of inorganic phosphorus increases (cf., Mueller and Hastings, '51). This condition made it necessary for Mueller and Hastings ('51) to average consecutive determinations of the phosphorus concentration in order to make an approximation to the rates of phosphorus exchange in whole human blood during successive intervals. Their data calculated in this manner indicate large variations in the rates at which phosphorus entered and left the blood cells with no discernible trends during a three-hour period.

Since the ideal conditions in which the blood pH remained constant were difficult to realize, it was considered desirable in our previous investigations of the uptake of phosphate ions by human erythrocytes (Gourley, '52) to choose a condition in which the net movement of phosphate ions was from the plasma into the cells. This was accomplished by incubating the blood with air as the gas phase. During the first three hours of incubation CO_2 was liberated and the pH of the blood rose continuously. In this paper the rates of turnover of the plasma phosphate ions under these experimental conditions have been calculated for human blood and for blood of the rabbit and chicken.

METHODS

Human blood was drawn from healthy young males by venipuncture and the blood from rabbits and chickens (New Hampshire Reds) was drawn by cardiac puncture. For experiments on rabbit or chicken blood, samples from two animals were pooled. Crystalline heparin was used as the anticoagulant. A stoppered flask containing the blood and sufficient isosmotic (0.3 M) glucose solution to increase the concentration by 200 mg of glucose per 100 ml was mounted in a Warburg water bath regulated at 37.0 ± 0.02°C. and shaken at 60 to 70 oscillations per minute. After temperature equilibration, 0.2 to 0.4 ml of a solution containing 1 to 2 μg P as Na_2HPO_4 as carrier and approximately 25 μc P^{32} in the form of dilute H_3PO_4 in 0.9% NaCl was added at zero time.

At various time intervals an aliquot of about 1 ml was removed for the determination of the hematocrit in a Wintrobe tube (centrifuged one hour at 3000 r.p.m.). An aliquot of 6 to 7 ml was also removed, chilled in crushed ice and centrifuged immediately at 0°C. Exactly 3.0 ml of the plasma so obtained was added to 10 ml 15% trichloroacetic acid (TCA). The protein precipitate was stirred at 0°C., the supernatant removed after centrifugation, and the precipitate washed once with 3 ml 5% TCA. The supernatant and washings of the plasma samples were made up to 25.0 ml. On suitable aliquots the inorganic phosphorus concentration was determined by the Fiske-Subba-Row method and the radioactivity determined by means of samples dried on small aluminum dishes as previously described (Pertzoff and Gemmill, '49). The samples were counted at a fixed distance from a thin glass wall beta counter to 2×10^4 counts. The radioactivity was usually sufficient to give counts of about 50 times the background, thus reducing the counting error to less than ± 2%.

RESULTS

Rabbit blood. The uptake of phosphate ions by rabbit erythrocytes under the conditions of these experiments was found to agree closely with that described by Hahn and Hevesy ('42) and Lovegrove, DeLuca and Rossiter ('52). Since rabbit blood containing a high percentage of reticulocytes takes up labeled phosphate ions more rapidly than normal cell suspensions (Lovegrove et al., '52) each rabbit was bled only once for this series of experiments. It was assumed that the reticulocyte count was therefore in the normal range. The average P^{32} uptake in 120 minutes was 53 ± 4% (mean and standard deviation of the mean of determinations of 5 samples, average hematocrit = 36%).

To estimate the turnover rate during the initial period of phosphate uptake the conventional plot of the relative specific activity (R.S.A.) of the plasma inorganic phosphate on a logarithmic scale against time was made. In figure 1 each solid circle is the average of determinations in 5 experiments. These points fall reasonably close to a straight line. By extrapolation of this straight line, the half-value time of the phosphate turnover (220 min.) was obtained. The half-value time ($t\frac{1}{2}$) is related to the turnover rate (k) by the equation $k = 0.693/t\frac{1}{2}$ (Siri, '49). Thus for an average $t\frac{1}{2} = 220$ min. the turnover

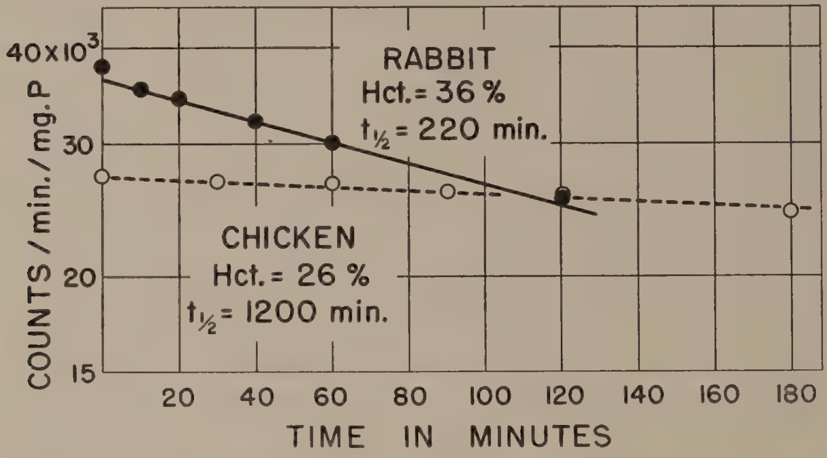

Fig. 1 A semi-logarithmic plot of the relative specific activity of the plasma inorganic phosphate of rabbit and chicken blood against time. The solid line represents the average of the data from 5 experiments on rabbit blood. The broken line represents the average of the data from two experiments on chicken blood. The relative specific activity is calculated as the counts per minute per milligram plasma P per 10^5 counts per minute added per 100 ml blood. The half-value times ($t\frac{1}{2}$) determined from the graph by inspection have been used to calculate the turnover rates as described in the text.

rate, expressed as a per cent, is 0.32% of the plasma phosphate per minute. The corresponding values in individual experiments appear in table 1.

Chicken blood. Hahn and Hevesy ('42) reported that the uptake of labeled phosphate ions by the nucleated erythrocytes of the chicken was considerably slower than the corresponding uptake by mammalian erythrocytes. This result was confirmed in the present study. In two experiments in which the average hematocrit was 26%, less than 5% of the added P^{32} had entered the cells after incubation for three hours. A rough estimate of the turnover rate can be made in the manner already described for rabbit blood. The average data for the two experiments

TABLE 1

The half-value times and turnover rates of phosphate exchange in rabbit and human blood

SPECIES	EXPT. NO.	HCT.	HALF-VALUE TIME, MIN.		TURNOVER RATE, % PER MIN.	
			Fast phase	Slow phase	Fast phase	Slow phase
		%				
Rabbit	27	39		190		0.36
	29	33		190		0.37
	32	36		230		0.30
	34	36		210		0.33
	37	36		280		0.25
	Mean	36 ± 2		220 ± 40		0.32 ± 0.05
Human	18	46	10	350	6.9	0.20
	19	46	10	320	6.9	0.22
	31	47	10	360	6.9	0.19
	35	48	12	260	5.8	0.27
	47	47	5	230	14	0.31
	48	42	6	290	12	0.24
	49	45	7	330	9.9	0.21
	52	44	7	290	9.9	0.24
	51[1]	46	4	110	17	0.65
	Mean	46 ± 2	8 ± 3	300 ± 45	9.0 ± 2.8	0.24 ± 0.04

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[1] Equilibrated with a gas phase of 5% CO_2 and 95% O_2. This experiment is not included in the means.

have been plotted in figure 1 (open circles, broken line). An extrapolation of the straight line joining the points gives an approximate half-value time of 1200 min. From this value the turnover rate is calculated to be about 0.058% of the plasma phosphate per minute.

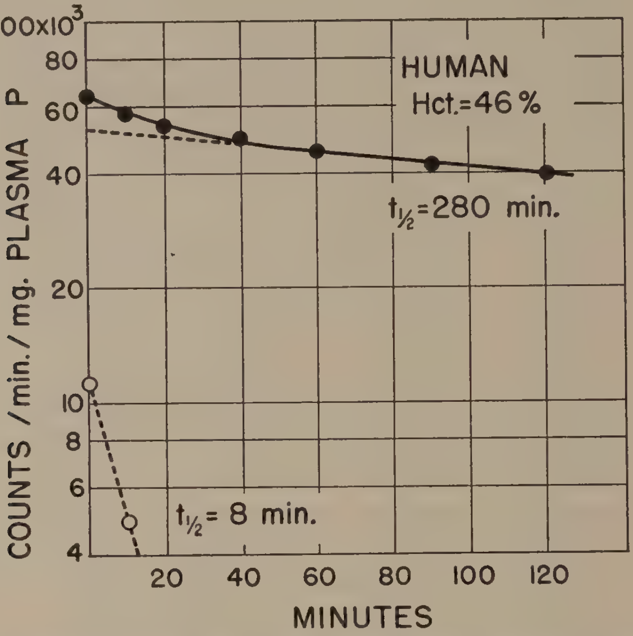

Fig. 2 A semi-logarithmic plot of the relative specific activity of the plasma inorganic phosphate of human blood against time. The data from 8 experiments have been averaged. The observed values are shown as closed circles (solid line). The experimental curve has been resolved into two straight line components (broken lines) as described in the text.

Human blood. The results of 8 experiments in which human blood was equilibrated with air and one experiment in which blood was equilibrated with 5% CO_2 and 95% O_2 appear in table 1. The average results of the 8 comparable experiments are shown in figure 2. It will be seen in figure 2 that a semi-logarithmic plot of the R.S.A. of the plasma phosphate against time (solid line) produces a curve rather than a single straight line. The fact that the R.S.A. of the plasma inorganic phosphate fell more rapidly during the first 40 min. than subsequently suggests that there were two or more simultaneous processes by which P^{32} was removed from human plasma. Since the second part of the curve approximates a straight line, it can be extrapolated back to zero time and this extrapolated value subtracted from the first part of the experimental curve. A plot of the resulting values (broken line) gives the true rate of the initial process (Siri, '49). The initial process by which phosphate ions leave the plasma is apparently very rapid, having a half-value time of 8 min. and a turnover rate of 9% per minute. The second, slower phase has a half-value time of about 300 min. and a turnover rate of 0.24% of the plasma phosphate per minute.

DISCUSSION

A two-phase disappearance of a substance from plasma is frequently observed in tracer experiments *in vivo*. It is usually assumed that the injected tracer material quickly comes into diffusion equilibrium with the interstitial fluid of the body tissues. This results in an initial rapid loss of the tracer from the plasma followed by a slower loss characteristic of the metabolic process under study. It is rare however to observe a similar result in tracer experiments *in vitro* and obviously such a result, when observed, must be explained on some other basis. Falkenheim, Underwood and Hodge ('51) found that the disappearance of P^{32} from the medium when shaken with powdered bone indicated that the uptake of the labeled phosphate occurred in two phases. In this case the initial rapid reaction was interpreted as exchange at the surface of the bone particles while the slower reaction was interpreted as characteristic of the recrystallization process. Sheppard, Martin and Beyl ('51) found that the disappearance of Na^{24} from human blood plasma (but not from bovine, canine or sheep plasma) also indicated a rapidly exchanging and a slowly exchanging fraction in human erythrocytes.

It will be noted in table 1 (expt. 51) that the two-phase disappearance of P^{32} from human plasma still occurred even under a condition in which the usual pH shift in these experiments would be reversed. That is to say, with a gas phase of 5% CO_2 and 95% O_2, the pH would fall rather than rise progressively during the experimental period. This would seem to eliminate the possibility that the type of curve shown in figure 2 was an artefact attributable to a pH change. In the presence of 5% CO_2 and 95% O_2 there were apparently still two or more processes by which phosphate ions were exchanging although the turnover rates were greater under these conditions.

The curve shown in figure 2 might be explainable if the phosphate ions which left the plasma moved into the erythrocytes at one rate and into the remaining blood cells at a different rate. It was previously found (Gourley and Gemmill, '50) that removal of the white cells and platelets did not significantly affect the P^{32} uptake by human blood so it is doubtful that the various cells take up P^{32} at markedly different rates. Another explanation might be that there is an initial rapid surface exchange adsorption of phosphate ions on the cell surfaces, followed by the slower active transport of the ions into the cells. This hypothesis is only acceptable if one assumes that such an adsorption occurs on human erythrocytes but not on rabbit or chicken erythrocytes during the period of observation.

There is an alternative to the above attempts to explain the type of experimental curve in figure 2 which appears more reasonable. Such a curve would result if there were two or more intracellular compartments into which phosphate ions entered at different rates. A complete understanding of the nature of these compartments must await further experimentation. It is possible only to speculate on their nature at the present time. Although only one compound, adenosine triphosphate (ATP), appears to have the prerequisites for being the precursor of intracellular inorganic phosphate, the possibility that some inorganic phosphate may diffuse into human erythrocytes has not been excluded (Gourley, '52). Furthermore, diffusion of phosphate into human erythrocytes was observed in the presence of 2×10^{-2} M sodium fluoride which at the same time inhibited the slower active phosphate uptake (Gourley, '51). It seems conceivable therefore that even under normal circumstances there is an identifiable initial rapid diffusion of phosphate ions into a minor compartment of the erythrocytes in addition to the slower chemical transfer into a major compartment. Either this minor compartment does not exist in the blood cells of other species investigated or else it is so small that equilibrium is reached very quickly and its rate cannot be measured by this technique. Examination of figure 1 suggests that the latter may actually occur in rabbit blood since the point at zero time lies slightly above the straight line. However too much significance should not be attached to this particular point because it was calculated on the assumption that there was a negligible change in the concentration of inorganic phosphate in the plasma during the first 10 min. Actually there is a slight drop in plasma phosphate concentration during this interval so the elevation of the point at zero time can be at least partly accounted for.

If this tentative explanation of the initial rapid uptake is accepted, the second slower phase of phosphate uptake by human erythrocytes is presumably characteristic of the active transport process previously postulated (Gourley, '52). It is interesting to note that the mean turnover rate for this phase of phosphate uptake in human erythrocytes (0.24% per min.) is of the same order as the mean turnover rate in rabbit erythrocytes (0.32% per min.) although the difference is statistically significant. It was concluded earlier that phosphate ions enter human erythrocytes by the formation of ATP at the cell membrane (Gourley, '52). This same mechanism may operate in rabbit erythrocytes because rabbit blood cells typically contain higher concentrations of ATP than human blood cells. In the present experiments the rabbit blood with its lower hematocrit contained approximately the same amount of ATP as the human blood with its higher hematocrit. This may possibly account for the similarity in phosphate turnover rates in the two species in spite of the 10% difference in cell volumes.

The slow rate of phosphate turnover in chicken erythrocytes is undoubtedly related to the type of metabolic reactions which predominates in these cells. Whereas in mammalian erythrocytes the energy-producing reactions appear to be mainly glycolytic, in avian erythrocytes oxidative processes ordinarily produce all of the energy (Ponder, '48). It is possible that none of the oxidative reactions involving phosphate ions occur at the membrane of the chicken erythrocyte but occur at sites inside the cell utilizing mainly intracellular inorganic phosphate.

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SUMMARY

The rates of exchange of phosphate ions have been determined in human, rabbit and chicken blood under *in vitro* conditions in which the net movement of phosphate ions was from the plasma into the cells. The turnover rate was 0.32% of the plasma phosphate per minute in rabbit blood and 0.058% per minute in chicken blood. In human blood the uptake of phosphate ions occurs in at least two phases. It is suggested that the initial rapid phase (turnover rate = 9% per minute) represents diffusion of phosphate ions into the blood cells while the slower phase (turnover rate = 0.24% per minute) is characteristic of the active transport of the ions into the cells of human blood.

LITERATURE CITED

FALKENHEIM, M., E. E. UNDERWOOD AND H. C. HODGE 1951 Calcium exchange; the mechanism of adsorption by bone of Ca^{45}. J. Biol. Chem., *188:* 805–817.

GOURLEY, D. R. H. 1951 Inhibition of uptake of radioactive phosphate by human erythrocytes *in vitro*. Am. J. Physiol., *164:* 213–220.

——— 1952 The role of adenosine triphosphate in the transport of phosphate in the human erythrocyte. Arch. Biochem. Biophys., *40:* 1–12.

GOURLEY, D. R. H., AND C. L. GEMMILL 1950 The effect of temperature upon the uptake of radioactive phosphate by human erythrocytes in vitro. J. Cell. and Comp. Physiol., *35:* 341–352.

HAHN, L., AND G. HEVESY 1942 Rate of penetration of ions into erythrocytes. Acta Physiol. Scand., *3:* 193–223.

LOVEGROVE, T. D., H. A. DELUCA AND R. J. ROSSITER 1952 The *in vitro* incorporation of radioactive phosphate into rabbit reticulocytes. Can. J. Med. Sciences, *30:* 143–150.

MUELLER, C. B., AND A. B. HASTINGS 1951 The rate of transfer of phosphorus across the red blood cell membrane. J. Biol. Chem., *189:* 869–879.

PERTZOFF, V., AND C. L. GEMMILL 1949 The effect of anesthetics on the uptake of radioactive phosphorus by human erythrocytes. J. Pharm. and Exp. Therap., *95:* 106–115.

PONDER, E. 1948 Hemolysis and related phenomena. Grune and Stratton, Inc., New York. Appendix IV, 357–370.

RAKER, J. W., I. M. TAYLOR, J. M. WELLER AND A. B. HASTINGS 1950 Rate of potassium exchange of the human erythrocyte. J. Gen. Physiol., *33:* 691–702.

RAPOPORT, S., AND G. M. GUEST 1939 The decomposition of disphosphoglycerate in acidified blood: its relationship to reactions of the glycolytic cycle. J. Biol. Chem., *129:* 781–790.

SHEPPARD, C. W., AND W. R. MARTIN 1950 Cation exchange between cells and plasma of mammalian blood. I. Methods and application to potassium exchange in human blood. J. Gen. Physiol., *33:* 703–722.

SHEPPARD, C. W., W. R. MARTIN AND G. BEYL 1951 Cation exchange between cells and plasma of mammalian blood. II. Sodium and potassium exchange in the sheep, dog, cow, and man and the effect of varying the plasma potassium concentration. J. Gen. Physiol., *34:* 411–429.

SIRI, W. E. 1949 Isotopic tracers and nuclear radiations with applications to biology and medicine. McGraw-Hill Book Co., Inc., New York. Chap. *15*, 388–402.

SOME EFFECTS OF NaCl CONCENTRATION ON THE DEVELOPMENT OF EARLY CHICK BLASTODERMS IN CULTURE

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SIX FIGURES

The total molar concentration of a saline medium isotonic to the body fluids of early embryos has received little study from the biological point of view. Nevertheless the unanimous evidence that the freezing point of egg white is of the order of — 0.43°C., whereas hens' blood freezes at — 0.56°C., indicates a considerable difference in the activity of the water in the two fluids (literature discussed by Howard, '44). The freezing point of egg yolk, when determined with appropriate precautions, appears to be the same as that of egg white (Howard, '44), although the high viscosity, low water content and complex constitution of egg yolk leave the interpretation of freezing point studies necessarily tentative unless sustained by other evidence. However, regardless of the final decision as to the activity of the water in egg yolk, it is clear that the chick blastoderm, separated from the white only by the non-cellular vitelline membrane, is to some extent under the influence of the activity of the water in the white. Nevertheless embryologists frequently employ the conventional Ringer or Tyrode solution in studies on early chick embryos.

In the case of R. pipiens and other amphibia, Holtfreter ('43) has presented evidence that isolated pregastrula cells are in functional equilibrium with a balanced saline of 0.385% salts (NaCl 3.5, KCl 0.05, $CaCl_2$ 0.1, and $NaHCO_3$ 0.2 gm per liter), in contrast to the whole egg which is normally laid in fresh water and has a protective coating. In 0.385% saline single cells or fragments of embryos of various stages were kept alive for weeks, exhibited amoeboid movements, and underwent cell division and differentiation, whereas in 0.57% salts the ability of the cells to aggregate was reduced, cell division met with difficulties and prolonged culture was impossible. Nevertheless the vapor pressure of adult frog blood corresponds to that of 0.66% NaCl (Walker, '30), hence one may infer that in the frog the embryonic cells are functionally adjusted to a medium which is more dilute than that which surrounds adult cells.

Pannett and Compton ('24) recommended a salt solution for the culture of chick embryos in which the constituents were approximately equal to their concentrations in mammalian plasma with the exception of $NaHCO_3$. This constituent was omitted for convenience in sterilization, and the total molarities of other constituents equal that of a uni-univalent salt solution of 0.101 molar, which is slightly more dilute than egg white. This saline, in combination with minced chick embryo extract and fowl plasma, has been used successfully as a culture medium in several laboratories (Pannett and Compton, '24; Strangeways and Fell, '26; Waddington, '32; and Spratt, '40 and '42). It is not possible to evaluate precisely the osmolar contribution of the embryonic extract, but the final medium was probably considerably more dilute than adult plasma with respect to inorganic constituents. In a review of the application of tissue culture *in vitro* to embryology, Fell ('40) states that either Tyrode's or Pannett and Compton's salines are used in making embryonic extracts, but in various earlier papers does not state specifically which saline was used. Much of Fell's work has been done with considerably older embryos than are considered in the present study. Spratt later ('47) in the course of his studies on the development of the chick blastoderm on synthetic media, recommended a chick Ringer solution equivalent to 0.163 molar uni-univalent salt, but has not reported any specific study of the effects of changes in the concentration of the inorganic constituents in the culture media.

Murray ('34) studied the contractility of chick cardiac tissue in culture in a considerable range of saline concentrations. From his work it appears that the embryonic heart must be quite rugged in that it appeared to withstand large variations in the medium. He noted that the average frequency of the heart beat declined progressively when NaCl solutions of 0.8, 1 and 1.3% were used in combinations of 5 parts of salt solutions to one part of plasma.

The present experiments were designed to test specifically the effects on the development of whole chick blastoderms of media made up to total concentrations equivalent to: *(a)* egg white, and *(b)* hen blood. In addition, some data on the effects of lower concentrations are presented.

METHODS

The NaCl equivalent of egg white was taken as 123 mmolar, corresponding to a freezing point of — 0.425°C. (Howard, '32). The freezing points of the blood of a group of 4 hens were observed to range from — 0.562 to — 0.590°C. (Howard, '33). The higher values were found on sera from blood drawn under amytal anesthesia, the lower on heparinized blood drawn from quiet unanesthetized hens. These values may be compared with data on human blood which presumably had been drawn under more nearly resting conditions than obtained in studies of the blood of other mammals and of birds which often yield somewhat higher values. Gram ('24) reports an average value for human sera of — 0.562°C., range — 0.570 to — 0.550, and Fremont-Smith et al. ('31) observed, on 35 subjects, an average of — 0.567, range — 0.536 to — 0.593. Ball ('30) reported a value of — 0.561°C. for dog serum. Since the values for hen's blood fall within the range of the much more extensive data on the human, the value of — 0.560°C. has been taken as a round figure for hen's blood; possibly it is slightly on the low side of the mean. This was taken as corresponding to an NaCl solution of 163.1 mmolar.

Saline-agar-egg white media were used, following the general procedures of Spratt ('47). The final media contained 15% egg white, which contributes some metabolic substrates, buffers, and such bacteriostatic qualities that asepsis is unnecessary in short term studies. The 15 parts of egg white were combined with 85 parts of saline made up to contain 5 mmols of KCl and 1.6 mmols of $CaCl_2$ with the NaCl concentration adjusted so that the final osmolar concentration was as specified. Thus all constituents except NaCl were constant unless otherwise mentioned. Difco "special agar Noble" was added in a concentration of 0.32%. During the latter half of the work, solutions were made up in water redistilled once in glass. This precaution did not make any notable difference in the behaviour of the cultures.

There is a small amount of water loss from the media during incubation at 37.5°C. Using plate glass embryological watch glasses with a plane contact surface for the lid, the weight loss was less than 0.1% during incubation for 20 hours. When watch glasses with a molded surface which did not make such good contact with the cover were used, the weight loss under the same conditions was 0.4 to 1%. Both types of containers were used without any notable difference in the development of the cultures. The watch glasses were placed in a shallow brass chamber containing moist cotton and covered with a loosely fitting brass lid. The chamber was placed underneath a 15 watt bulb in the incubator so that its upper surface was somewhat warmer than its lower surface. This prevented distillation of water from the media on to the under surface of the watch glass cover.

Since, with the technique used, weight losses of 0.1 to 1% occurred in the incubator, and since there would presumably be somewhat faster drying of the surface than of the underlying agar, it seemed preferable to use a concentration slightly under the 163 mmolar blood equivalent, in order to avoid any hypertonicity in relation to adult blood level. Freezing point measurements of the final agar-egg white-saline medium showed that the combined effects of concentration during boiling of the agar-saline, evaporation in the watch glasses, and any contribution by the agar, did not exceed an equivalent of 3 mmols of NaCl. Accordingly the media were made up to 160 mmols, a value slightly under the 163 mmolar level, which itself is on the low side of the mean for hen's blood. The media of lower concentration were simply made up to the concentrations as specified. Cultures were incubated at 37.5 ± 0.5°C.

Fertile eggs were obtained from a commercial source, and were used one to 6 days after being received. Except when the egg appeared non-fertile or the embryo grossly retarded, blastoderms from alternate eggs were placed on 123 and 160 mm. media. Some difference in the performance of the embryos at different times was apparent, but in all cases the results on the two media are comparable: all other factors having been kept uniform as far as possible. Eggs were opened in the saline corresponding to the medium to be used, the blastoderm, dissected free from the vitelline membrane, was transferred to a second dish of saline for rinsing, and then placed on the agar surface which had been previously flooded with saline. Thus the blastoderm was always kept immersed in liquid. The ventral surface of the embryo was placed toward the agar, and all free saline cautiously removed by holding a gauze sponge against the edge of the agar. This causes the embryo to flatten out and adhere to the surface of the agar, which is finally covered only by a minimal film of liquid. Glass needles and a small plastic spoon or wide mouthed pipette were used in handling the blastoderm. In all stages through the head-process stage, the whole blastoderm was used. In some of the headfold stages the outer part of the zona opaca was trimmed away in order to prevent a tendency not to lie flat, a precaution which may not have been necessary. The zona opaca always carried some adherent yolk onto the agar.

Since the present study was designed to evaluate the effects of one variable, NaCl concentration, a simple medium has been used which permitted a degree of development approximating the normal in several respects, for some 20 to 40 hours after explantation. The medium used is obviously most incomplete in that it lacks yolk, except for the small amount carried onto its surface by the blastoderm, but it permits sufficient development to make possible an evaluation of some effects of variations in its ionic constituents.

RESULTS

The degree of development attained in culture depends to a considerable extent on the stage at explantation, earlier stages generally developing less well than later ones, with respect both to maximal size attained and degree of differentiation. The stages explanted were: *(a)* the unincubated blastoderm; *(b)* the early streak, after about 16 hours incubation *in ovo* at 37.5°C.; *(c)* the definitive streak, corresponding to H and H stage 4 (Hamburger and Hamilton, '51); *(d)* the head-process stage, H and H 5; and *(e)* the headfold stage, H and H 6. In these presomite stages the embryonic area consists of one to three cell layers, thicker in some areas than in others, but normally dependent on diffusion for its supplies of oxygen and substrates. Since circulation normally begins at the 16-somite stage (45 to 48 hours), these cultures constitute tissues whose chemical environment can be manipulated to a considerable extent without altering normal cellular interrelationships or having to resort to perfusion techniques to avoid subjecting the cells to unphysiological degrees of anoxia or substrate deprivation.

Morphogenesis was evaluated by comparing the condition of the explant with the photographs of normal stages published by Hamburger and Hamilton ('51). The degree of normality was usually not complete, but embryos were graded according to general body form, and given a number corresponding to the normal stage to which they most nearly approached, as illustrated in the photographs, where the embryo in figure 3 was classified as an H and H 10, and that of figure 5 as an H and H 12. With both saline levels the central nervous system was often small or poorly developed in relation to general body form. The brain frequently appeared to do less well than the heart in these explants, and presumably requires some nutrient or protection not furnished with the technique employed.

When the general body form did not approximate a normal pattern, it was not given a numerical score, and contributed zero to the averages of general morphogenesis given in table 2. In such cases there was frequently some degree of development, including the appearance of longitudinal axial tissue and bilateral symmetry. Many of these cases developed somites or pulsating hearts or both, so that their development contributes to the evaluation of the total picture under these headings. The somite count is a relatively objective measure of development, but even here the situation is only partly reflected in the count, as can be seen in the photographs. The embryo of figure 5, on 123 mM., has 17 somites, while the embryo of figure 6, on 160 mM. medium, has 16 somites, although the difference in size and regularity of somite development is obvious. However, embryos with a larger number of somites usually had somites which were more regular in form and more clearly outlined than embryos of a comparable stage at explantation with fewer somites. Thus quantitatively poor somite development tended to be associated with qualitatively poor development, as illustrated in figures 1, 2, 3, and 4. In many cases certain features, such as the eye or the auditory vesicle, were considerably ahead of general body form in the degree of devopment which was finally attained. Embryos often underwent some degree of flexion and torsion, but pre-somite explants were not observed to develop in general form beyond a point intermediate between H and H stages 13 and 14, corresponding to 19 to 22 somites, although the actual somite count frequently exceeded this figure.

The degree of development of the heart was evaluated according to size, vigor of beat and normality of form and position, in arbitrary ratings of one to 4 points. The variations in the vigor of beating were often extreme. Averages of these evaluations are listed in table 2 under the heading *Condition of the heart.* If no area of pulsation was noted, the rating was zero. The activity of the heart, in embryos in good condition, is very conspicuous, and has been particularly studied as an index of the physiological condition of the cultures.

The pulse rate constitutes a useful objective indicator of the condition of the heart. Pulse rates in the cultures are markedly influenced by temperature. In order to minimize variability due to temperature fluctuations, the procedure was, on removing the culture from the incubator for examination, to place the culture immediately on a warm stage, at 37.5°C., and to count the pulse immediately, without removing the cover glass, using a stopwatch and transmitted light. Counts during the first 20 seconds could usually be reproduced during the second 20 seconds, within 2% or better, unless the heart was obviously in poor condition and beating irregularly. When the cover glass was later removed for examination of morphological features, the pulse invariably slowed, presumably due to cooling of the surface of the culture. Too strong light was sometimes associated with warming and a subsequent acceleration, but in all cases pulse rates recorded were those counted immediately on removal from the incubator.

The pulse in any one heart generally rose to a maximum 40 to 50 hours after development had started, and thereafter declined until viability was lost. In table 2 are listed averages of the maximum pulses observed in embryos which showed pulsations. In general, the larger more vigorous hearts exhibited the highest pulse rates, and maintained these rates for 12 hours or more. Usually the more vigorous hearts maintained contractility longer than did the feeble hearts. Since small and feeble hearts sometimes, however, exhibited very rapid rates, pulse rate is not an uncomplicated index of cardiac condition in cultures. It will be noted, however, that the average maximum pulse rates parallel the other indices of developmental status. The rates observed in the cultures may be compared with the following figures for second day embryos *in ovo*. Bogue ('32) obtaining electrocardiograms on unopened eggs kept in a warm chamber (exact temperature unspecified), reported an average pulse rate for second day embryos of 149 per minute, range 120 to 170, for three day embryos 173, range 165 to 210. Cohn ('28) observed a rate of 182 per minute in three day embryos at 38°C., with a "very small" opening in the shell, while Romanoff and Sochen ('36), after removing a square inch of shell at the large end of the egg, report a rate of 111 per minute for second day embryos at 37.5°C., and 156 for three day embryos.

Whole blastoderms of unincubated eggs, discs of the order of 4 to 5 mm in diameter, and one to three cell layers in thickness, are normally adherent to the yolk only on the zona opaca, the pellucid area being already cleanly separated from the underlying yolk by a film of clear fluid, in the subgerminal cavity. When explanted without previous incubation, and cultured on agar for 40 to 60 hours at 38°C., some development occurred on the 123 mmolar, and considerably less on the 160 mmolar media, as indicated in tables 1 and 2. The size of structures was generally less than that attained by explants of streak and later stages. On the 123 mM. media the neural tube appeared in 91% of explants. The tube generally exhibited enlargement of the forebrain, and, in 41%, some separation into the three primary brain vesicles. The outlines of the head and somite regions approximated those of a 10 somite embryo in 77%, but the number of somites generally lagged considerably behind what might have been expected from the contours of the brain and general body form. Frequently the somite ridges appeared but the division into somites occurred only in the anterior part. Hensen's node was not conspicuous. On the other hand, in 160 mM. media recognizable neural tubes developed in only 25%, and none went as far as division into the three primary vesicles. An approximation of the contour of a normal 10 somite embryo occurred only in one of 20 explants on 160 mM., although some development of a round head and ''tail' was seen in three of the 20. In only two of the 20 was there separation of somites, and 60% showed no differentiation whatever on 160 mM. media.

TABLE 1

Effects of concentration of the medium on the frequency of incidence of certain criteria of development in 142 explanted chick embryos

STAGE EXPLANTED	MEDIUM	NORMAL FORM	SOMITES	PULSATING HEART	CIRCULATION OF BLOOD
	millimols	%	%	%	%
Unincubated	123	77	87	91	9
Unincubated	160	5	10	30	0
Early streak	123	91	100	91	36
Early streak	160	27	45	18	9
Defin. streak	123	82	100	97	50
Defin. streak	160	40	92	76	8
H. P. & H. F.	123	100	100	100	77
H. P. & H. F.	160	92	100	92	0

Pulsatile hearts developed in 91% of the explants from unincubated eggs cultured on the 123 mM. media, but pulsation occurred in only 30% of those on 160 mM. When pulsatile hearts did develop on 160 mM. media they were smaller, more feeble, and did not continue beating as long as did those on the 160 mM. media. Hearts on 123 mM. were often quite vigorous and in some cases continued beating for over 50 hours. In two cases active circulation between the embryo and the blastoderm developed. The results are summarized in the tables, where they may be compared with the results on explants of later stages. It appears clear that, under the conditions of these experiments, the 123 mM. medium is very much more favorable than is the 160 mM. for the cultivation of unincubated blastoderms.

Embryos explanted after 16 to 24 hours incubation *in ovo* undergo more development than do the explants of unincubated blastoderms. On the 123 mM. media, embryos are produced which frequently conform fairly well to the normal up to the 16 somite stage, with respect to general contours, somite development, regression of Hensen's node, and development of the heart and circulation. In the photographs, examples of three pairs of embryos are presented, one pair explanted at an early primitive streak stage, a second pair at the definitive streak stage, and the third pair at the headfold stage: they were photographed after 21 hours in culture. One member of each pair was grown on the 123 mM., the other on the 160 mM. medium. The differences are typical insofar as material which exhibits considerable variation can be typified by individual examples. In the present study, 60 embryos were explanted in preliminary observations while the technique was being developed. These behaved similarly to the embryos listed in the tables, but are not included because the procedures used for evaluating their condition did not strictly correspond to those finally developed. Results on a series of 140 embryos are averaged in the tables, where it can be seen that in all stages studied there is an improvement in development on the 123 mM. as compared to the 160 mM. medium. The early streak stage was usually more advanced than H and H 3, but not as advanced as H and H 4, in that the groove in the streak and Hensen's node were not clearly visible. With this exception, the incubated stages corresponded closely to those of Hamburger and Hamilton ('51).

In table 1 the incidence of various features is compared on an all or nothing basis, while in table 2 development is evaluated in more detail. In table 1 it will be seen that in the unincubated group there is a very great difference betwen embryos exposed to the two different media in respect to whether any normal body form, any somites or any hearts develop at all. These differences are still large in the group of definitive streak explants, although tending to decline; but, by the head process stage we note somites in all cases.

The ability to develop an active circulation of blood between the embryo and the outlying area is a very marked difference, however, which is clearly in evidence in the head-process and headfold explants. Circulation was considered established only when there was true circular flow. The preliminary back and forth motion of the erythrocytes in the vessels, which some embryos exhibited without ever achieving a true flow from arteries to veins, was not regarded as indicative of an establishment of circulation. When flow becomes established it is a very striking phenomenon, for the embryo and the blastoderm are highly vascular in culture. Vigorous circulation has often been seen to continue for 12 hours, but has not been observed to last for 24 hours. The failure of the circulation is not due to the heart, which often continues to beat for many hours after the corpuscles have ceased to flow. In the few cases in which there was circula-

TABLE 2

Effects of concentration of the medium on the degree of development of explanted chick embryos

STAGE EXPLANTED	MEDIUM	NUMBER OF EMBRYOS	MORPHOGENESIS	SOMITE NUMBER	LENGTH	CONDITION OF HEART	PULSE PER MINUTE
	millimols				*mm*		
Unincubated	123	22	8.0	6.1	2.3	1.6	53
Unincubated	160	20	0.5	0.6	1.6	0.3	39
Early streak	123	11	9.9	16.2	2.9	2.6	75
Early streak	160	11	2.3	2.6	2.5	0.5	30
Defin. streak	123	28	9.6	16.8	3.3	3.0	75
Defin. streak	160	25	4.3	10.4	2.8	1.4	58
Head process	123	8	11.6	20.6	3.7	3.3	99
Head process	160	7	9.1	15.6	3.5	1.7	63
Head fold	123	5	12.6	21.8	4.6	3.8	118
Head fold	160	5	11.2	13.4	4.1	2.0	42

Length refers to total length of the embryo when a recognizable longitudinal axis was present.

tion in embryos on the 160 mM. medium, the flow was either much more sparse or became established many hours later than it did in embryos on the 123 mM. medium. A limiting factor in the persistence of the circulation may be that the blastoderm does not grow out on the surface of the agar as it does over the surface of the yolk *in ovo*. Some increase in the size of the embryos occurred during the period of culture. Maximum lengths attained are listed in table 2. It is clear that early explants do not achieve the length of later ones, so that there must be considerable limitation of the normal growth rate by the conditions of culture.

In table 2 it will be noted that, although the difference in general morphogenesis is gradually declining with the increase in the age at explantation, the average values of all features listed show a consistent difference in favor of the 123 mM. medium. Such morphogenesis as did occur on the 160 mM. medium was, as illustrated in the photographs, less perfect than on the 123 mM., and the vigor and rate of the heart were inferior.

Some observations were made on the effects of more dilute media, namely 76.5 and 101 mmolar, made up as described above, so that NaCl was the only variable. Cultures were always set up with alternate controls on 123 mM. In these experiments eggs were opened in 123 mmolar saline and thereafter the embryos were transferred to the saline corresponding to the medium to be used. The 101 mM. level is of interest since it corresponds to the total osmolar concentration of Pannet-Compton fluid.

On the 76.5 mM. medium the development in the cultures was very inferior to that on 123 mM. When definitive streak and head-process stages were explanted to 76.5 mM. media, there was, nevertheless, some development, consisting of the formation of a relatively small head, a long but narrow somite region and "tail," with regression of the streak. The structures were poorly outlined, the somites small and indistinct, although the total length of the 9 embryos on 76.5 mM. media averaged 3 mm, compared to 3.6 mm for their 9 controls on 123 mM. After 34 hours' culture, the somite count averaged 8.5 for the 9 on 76.5 mM. and 18.2 for the 9 on 123 mM. The hearts of these cultures on 123 mM. reached their maximum vigor and maximum pulse rates 30 to 34 hours after explantation. On 76.5 mM. pulsating hearts were never observed by 34 hours of culture, but did appear in 7 out of 9 cultures by 44 hours. The hearts on 76.5 mM. were always much smaller, much less vigorous, and beat at a somewhat slower rate, 73 per minute, than the hearts on 123 mM., which in this group averaged 110 per minute. The hearts on 76.5 mM. were frequently placed too far anteriorly, and usually did not emerge from underneath the embryo. It is uncertain whether the delayed appearance of pulsation represents a slowing of development with eventually some degree of adaptation to the dilute medium, or whether the embryonic tissues had by 44 hours built up a local environment differing from that of their medium. The cultures of streak stages on 123 mM. media usually showed signs of decreased vitality by 44 hours, so that the status of such cultures is highly dubious.

There was much less difference between 101 and 123 mM. than there was between 76.5 and 123 mM. Thus, no clear cut difference was observed in the morphogenesis of body form when explants of definitive streak and head-process stages on 101 mM. media were compared with alternate controls on 123 mM. The somite count averaged 19.8 in 17 cultures on 101 mM., and 17.8 in 17 cultures on 123 mM. media, a difference of doubtful significance. The central nervous system was highly variable at both NaCl levels.

In the case of the heart, on the other hand, on 123 mM. media there were 8 instances of well developed very vigorous hearts in the 14 of 17 cultures which developed pulsatile hearts, while on 101 mM. there were only two well developed very vigorous hearts in 17 cultures, although all 17 developed pulsatile hearts. The mean of the maximal pulse rates was 104 per minute on 123 mM. and only 86 on 101 mM. The circulation was also distinctly more abundant and vigorous on 123 mM. than on 101 mM. Ten cultures on each medium developed some circulation, but on 101 mM. there was often no more than a sparse trickle of cells: although there were large masses of erythrocytes in the outer regions of the area vasculosa, these masses were not usually in motion. When the circulation was maximally developed, there was rapid flow of large quantities of erythrocytes throughout the embryo and considerable areas of the highly vascular blastoderm. There were 6 instances of this maximal profuse circulation on 123 mM. media and only two instances on 101 mM.; and, on 123 mM. the circulation frequently continued for a longer time.

Thus it may be concluded that, with respect to the vigor of the heart and circulation, there is a definite superiority of 123 mM. media over 101 mM. There were, however, in general much fewer and less marked differences between the cultures on 123 and 101 mM. than between those on 123 and 160 mM.

DISCUSSION

In general the observations indicate that, with respect to a number of criteria, a more nearly normal development of presomite embryos occurs on the 123 mM. medium than on the 160 mM., and that, since the cultures on 101 and 76.5 mM. do less well, the 123 mM. medium approximates an optimum concentration. These findings are not unexpected, since the freezing point of egg white indicates that the activity of the water in this fluid corresponds to that in a 123 mmolar NaCl solution. Thus the evidence is clear that the 123 mM. concentration is equivalent in water activity to the normal environment of the dorsal surface of the embryo. The present findings that the embryos do markedly better in culture when their ventral surface is supplied with 123 instead of 160 mM. media is regarded as strong presumptive evidence that the interpretation of the freezing point studies previously advanced (Howard, '32, '44) does essentially describe the situation, and that the activity of the water in egg white and egg yolk is in fact identical.

A corollary of this identity is, however, that the early chick embryo is in functional equilibrium with a considerably higher water activity than are adult cells. This would be expected to have an influence on protein solubilities and presumably on enzyme reaction rates. A pronounced influence of NaCl and KCl, in the range between 0.1 and 1 molar, on the activity of various oxidases from mouse melanomas, has been described by Riley ('50), and a similar effect of NaCl concentration on a renal glutaminase was noted by Binkley ('50).

The observed effects on differentiation might well be construed as in part the results of influences of NaCl on reaction rates, either directly or by virtue of its influences on water activity. Thus, the slowing of the pulse at the higher NaCl level might be an effect on some limiting reaction rate, although probably not on an ultimate pacemaker reaction directly, since quite high pulse rates were sometimes observed in the presence of the higher NaCl concentration. In general, development did not proceed as rapidly or as completely on the 160 mM. as on the 123 mM. medium. Slowing of key reactions, or disruption of reaction sequences or couplings could conceivably explain many of the differences.

What is the biological significance of this adjustment of embryonic tissue to a medium of higher water activity than that of adult blood? Is the more dilute environment more favorable for biochemical reasons? Or, is the more dilute environment more favorable because it corresponds to the composition of the oceans at some critical period when the protoreptilian ancestry of the birds was being evolved? If so, this level of concentration might be taken to be indicative of the level of ocean concentration to which protoreptilian stock was adapted at some period prior to that at which it acquired a closed circulatory system and the ability to regulate its internal environment at the level used by the mature avian organism of the present. It will be of great interest to determine, if possible, at what developmental stage the transition to the adult level of water activity takes place.

SUMMARY

Pre-somite chick blastoderms of various stages were explanted to the surface of saline-agar-egg-white media made up to total molarities equivalent to: *(a)* that indicated by the freezing point of egg white, namely the equivalent of 123 mM. NaCl; and, *(b)* that of hen's blood, namely 160 mM., the only variable being the NaCl concentration. It was found that on the 123 mM. medium, more nearly normal development was obtained with respect to morphogenesis of general body form, number of somites, general form and vigor of the heart, and the establishment of blood circulation between the embryo and the area vasculosa.

On 101 mM. media, cultures were slightly less vigorous than on 123 mM., and on 76 mM. development was markedly inferior.

The observations are considered as evidence that the early chick embryo is normally adjusted to a water activity in its environment corresponding to that of a 123mM. NaCl solution, and that this presumably corresponds to the water activity in egg yolk as well as egg white. This is regarded as possibly an instance of biological preservation of a marine concentration characteristic of an epoch at which some critical biological adaptation took place.

ACKNOWLEDGMENTS

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LITERATURE CITED

Ball, E. G. 1930 The composition of pancreatic juice and blood serum as influenced by the injection of inorganic salts. J. Biol. Chem., *86:* 449.

Binkley, F. 1950 The role of glutaminase in tubular processes. First Macy Conference on renal function, p. 63, New York.

Bogue, J. Y. 1932 The heart rate of the developing chick. J. Exp. Biol., *9:* 351.

COHN, A. E. 1928 Chicken Embryos. XIII. The temperature characteristics for the contraction rate of the whole heart. J. Gen. Physiol., *11:* 369.

FELL, H. B. 1940 The application of tissue culture *in vitro* to embryology. J. Roy. Microscopical Society, *60:* 95.

GRAM, H. C. 1924 Composition and physical properties of normal human blood: a compilation from the literature. Am. J. Med. Sci., *168:* 511.

FREMONT-SMITH, F., G. W. THOMAS, M. E. DAILEY AND M. P. CARROLL 1931 The osmotic pressure (freezing point depression) of human cerebrospinal fluid and blood serum. Brain, *54:* 303.

HAMBURGER, V., AND H. E. HAMILTON 1951 A series of normal stages in the development of the chick embryo. J. Morph., *88:* 49.

HOWARD, EVELYN 1932 Osmotic relationships in the hen's egg, as determined by colligative properties of yolk and white. J. Gen. Physiol., *16:* 107.

——— 1933 The freezing point of the milieux of germ cells and the significance of their hypotonicity to the adult. J. Cell. and Comp. Physiol., *3:* 291.

——— 1944 Further cryoscopic evidence that the embryonic milieu is hypotonic to the adult. J. Cell. and Comp. Physiol., *24:* 201.

HOLTFRETER, J. 1943 Properties and functions of the surface coat in amphibian embryos. J. Exp. Zool., *93:* 251.

MURRAY, P. D. F. 1935 Fibrillation of the chick embryo heart *in vitro.* I. The effects of excess potassium, calcium, magnesium and sodium, and of high and low osmotic pressures. Proc. Roy. Soc. B, *116:* 434.

PANNETT, C. A., AND A. COMPTON 1924 The cultivation of tissues in saline embryonic juice. Lancet, *206:* 381.

ROMANOFF, A. L., AND M. SOCHEN 1936 Thermal effect on rate and duration of embryonic heart beat of Gallus Domesticus. Anat. Rec., *65:* 59.

RILEY, V. 1950 Salt inhibition and enhancement of cytochrome, succinic, and dopa oxidase systems of mouse melanomas. Proc. Soc. Exp. Biol. Med., *75:* 644.

SPRATT, N. T. 1940 An *in vitro* analysis of the organization of the eye forming area in the early chick blastoderm. J. Exp. Zool., *85:* 171.

——— 1942 Location of organ-specific regions and their relationship to the development of the primitive streak in the early chick blastoderm. J. Exp. Zool., *89:* 69.

——— 1947 A simple method for explanting and cultivating early chick embryos *in vitro.* Science, *106:* 452.

STRANGEWAYS, T. S. P., AND H. B. FELL 1926 Experimental studies on the differentiation of embryonic tissues growing *in vivo* and *in vitro.* I. The undifferentiated limb bud. Proc. Roy. Soc. B, *99:* 340.

WADDINGTON, C. H. 1932 Experiments on the cultivation of chick and duck embryos, cultivated *in vitro.* Phil. Trans. Roy. Soc. of London, *221 B:* 179.

WALKER, A. M. 1930 Comparisons of total molecular concentrations of glomerular urine and blood plasma from the frog and from *Necturus.* J. Biol. Chem., *87:* 499.

PLATES

PLATE 1

EXPLANATION OF FIGURES

Photographs of unstained living explants × 20

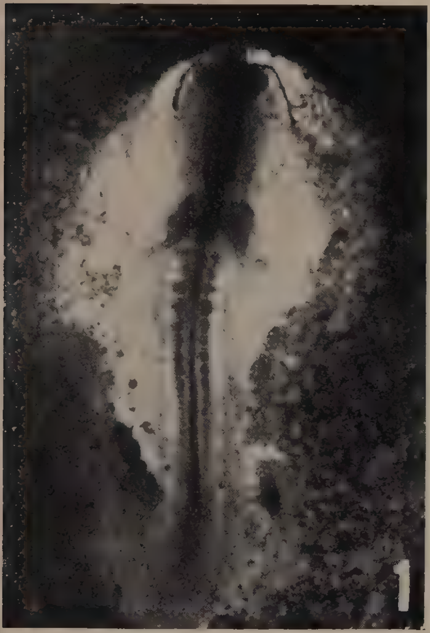
Photography by Chester Reather

1 *One hundred and twenty-three mM.* Embryo explanted on 123 mM. medium as a primitive ungrooved streak, blastoderm diameter 8 mm. Photographed after 21 hours' culture. Classified as H and H stage 9.7, having 9 somites, although some abnormalities are present. This specimen later developed 22 somites, a brain and head rated as H and H 11, and a moderately active heart, but no circulation.

2 *One hundred and sixty mM.* Embryo explanted on 160 mM. medium as a primitive ungrooved streak, having a blastoderm diameter of 8 mm, on same day as figure 1. Photographed after 21 hours' culture. Morphogenesis regarded as too atypical to receive a numerical score, although some development has occurred. Credited with 4 somites which are very poorly outlined. This specimen developed no further, and did not differentiate a pulsatile heart.

3 *One hundred and tweny-three mM.* Embryo explanted to 123 mM. medium as a definitive streak, H and H 4, blastoderm diameter being 8 mm. Photographed after 22 hours' culture. Classified as an H and H 10, having 10 somites, on the basis of general form, although the brain is poorly developed. This specimen later developed a very vigorous heart and 17 somites, but no circulation.

4 *One hundred and sixty mM.* Embryo explanted to 160 mM. medium, on same day as figure 3, as a definitive streak with beginning head-process, blastoderm diameter 10.5 mm, i.e. slightly more advanced than figure 3. Photographed after 22 hours' culture. Morphogenesis too poor to receive a numerical score, but credited with 8 somites. This specimen developed no further, and did not differentiate a pulsatile heart.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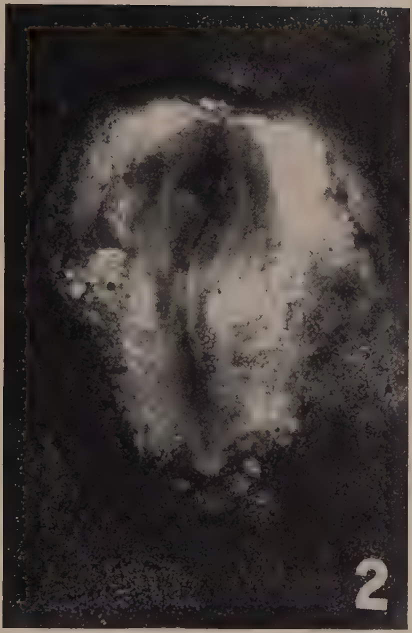
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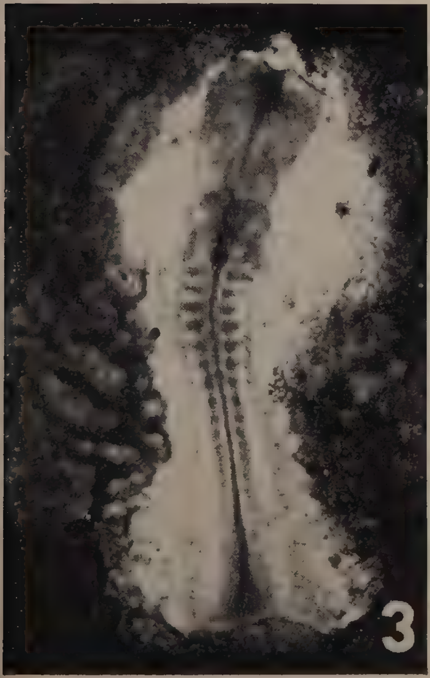
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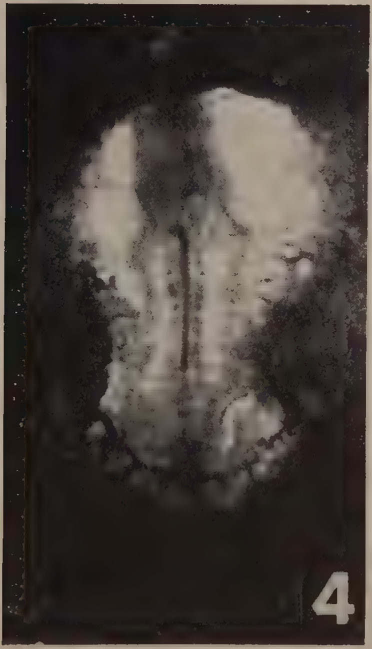
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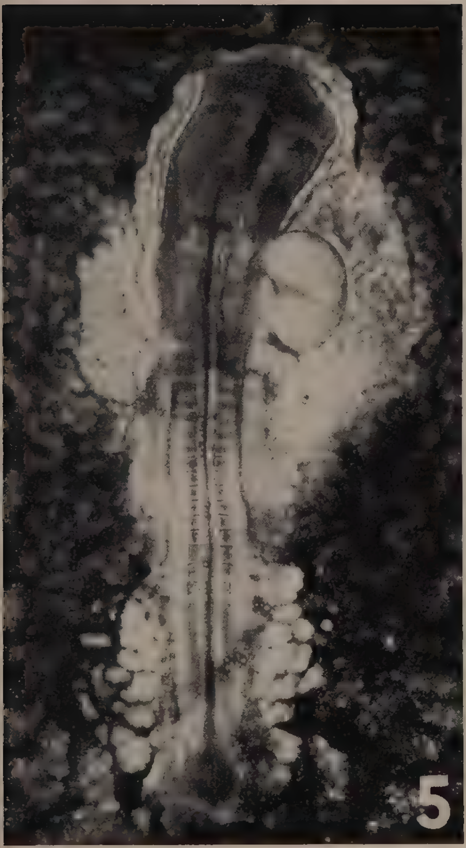
PLATE 2

EXPLANATION OF FIGURES

5 *One hundred and twenty-three mM.* Embryo explanted as an early headfold, H and H 6, without somites, blastoderm diameter being 11.5 mm. Photographed after 21 hours' culture. Classified as an H and H 12, although certain defects are present. It has 17 somites, a grade 4 heart which was beating vigorously at 103 per minute, producing a back and forth motion of erythrocytes in the vessels. Eight hours later a profuse circulation was established with a pulse of 144 per minute.

6 *One hundred and sixty mM.* Embryo explanted to 160 mM. medium, on same day as figure 5, as a headfold, H and H 6, without somites but slightly more advanced than the embryo of figure 5, and having a blastoderm diameter of 12.5 mm. Photographed after 21 hours of culture. Classified as an imperfect H and H 11. Although 16 somites are present, they are much less distinct and regular than those of figure 5, and the somite region, like the whole embryo, is smaller. The brain is small and less differentiated than in figure 5. The body outlines are characteristically complicated and disorderly, in contrast to the relatively clear outlines of figure 5. The grade 1 heart was beating feebly at a rate of 59 per minute. Eight hours later the pulse was only 13 per minute, and no circulation became established.



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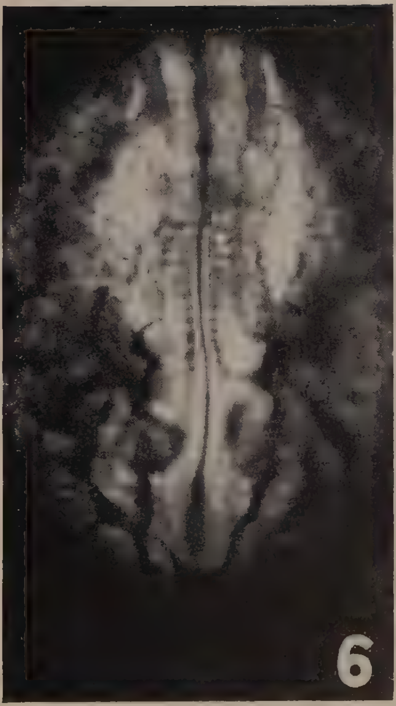

6

RESPIRATORY REFLEXES AND THE FLABELLUM OF LIMULUS[1]

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EIGHT FIGURES

Normal aerobic metabolism can be maintained by animals only when the partial pressures of oxygen and carbon dioxide at the respiratory surfaces lie within certain definite limits. In various organisms the scope of these limits and the period during which they may be transgressed without dire consequence, naturally depend on many physiological factors. Not the least interesting of these are the regulatory devices activated under emergency conditions, devices which tend to sustain normal gas exchange despite abnormal partial pressures of respiratory gases. Hyperventilation is the commonest of such mechanisms, but many other kinds of responses, such as specific searching and avoidance behavior, are involved in certain cases. It will be seen below that even inhibition of breathing is widespread in occurrence as an emergency measure adapted to cope with temporary respiratory stress.

Clearly, any regulatory device of this sort must include a means of detecting deviations from the normal partial pressures of oxygen and carbon dioxide. Such detectors have been found to be chemoreceptors which respond to excess or deficiency of respiratory gases. Their stimulation evokes reflex adjustments tending to restore the respiratory *status*

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[1] Aided by a grant from the American Academy of Arts and Sciences. A preliminary account of this work was presented to the American Society of Zoologists in December, 1951 (Waterman and Travis, '51).

quo. The chemoreceptors concerned may be exteroceptors such as those found in the first antennae of crabs, the gills and gill covers of crayfish, the stigmatal and spiracular regions of spiders and insects, and the upper respiratory tract of birds. They may be peripheral interoceptors like those of the *glomus aorticus* and the *glomus caroticus* of mammals. Or finally, they may actually be elements within the central nervous system as shown in particular cases for insects, crustaceans and vertebrates.

Quantitative measurements of the responses of such special respiratory sense organs have been made only in the vertebrates (Heymans and Rijlant, '33). All chemoreceptors of arthropods and other invertebrates have generally proved refractory to electrophysiological techniques (e.g., Dethier and Chadwick, '48) although positive results have recently been obtained for contact chemoreceptors in *Limulus* (Barber, '51).

The present report describes studies designed to elucidate these relationships in the horseshoe crab, *Limulus polyphemus* (L.).[2] Experiments of Hyde (1894, '06) and Patten ('12) long ago demonstrated that the whole respiratory mechanism of this animal deserved more thorough study. In a few experiments, Hyde ('06) found breathing in *Limulus* to be slowed by saturating the seawater with hydrogen and that no dyspnea resulted in CO_2 saturated water. These and other data led her to suggest that respiratory chemoreceptors were probably present in horseshoe crabs to test the external medium for respiratory gases. These ideas form one of the roots of the present research. It is striking that the challenge of these suggestive facts has been so long ignored despite *Limulus'* great interest to students of evolution and for all its fascination as an experimental animal.

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[2] Many invertebrate zoologists had accepted the claims of Pocock ('02) that Gronovius' (1764, p. 220) genus *Xiphosura* was the correct one for *Monoculus polyphemus* Linnaeus (1758, p. 634). However, the International Committee on Zoological Nomenclature (Opinion 104, '28) has officially declared *Limulus* O. F. Müller (1785, p. 126) a *nomen conservandum* for this animal, a decision which will hardly displease most textbook writers and physiologists who have been using it right along. More recently, further complications concerning the technically correct name for this well known form have arisen (Størmer, '51), but these would appear unlikely to change official sanction of the genus *Limulus*.

More specifically, the aims of this work were, first, to demonstrate *Limulus'* respiratory reflexes as they relate to partial pressures of oxygen and carbon dioxide; second, to begin a search for chemoreceptors concerned. The initial hypothesis tested relative to this second point was that the flabellum, a paired spatulate structure articulating with the base of the 5th walking legs (fig. 1) is a specific chemoreceptor concerned with the reflex regulation of breathing. However, it will be seen from what follows that neither the basic respiratory rhythm nor the respiratory reflexes so far found depend on the integrity of these organs.

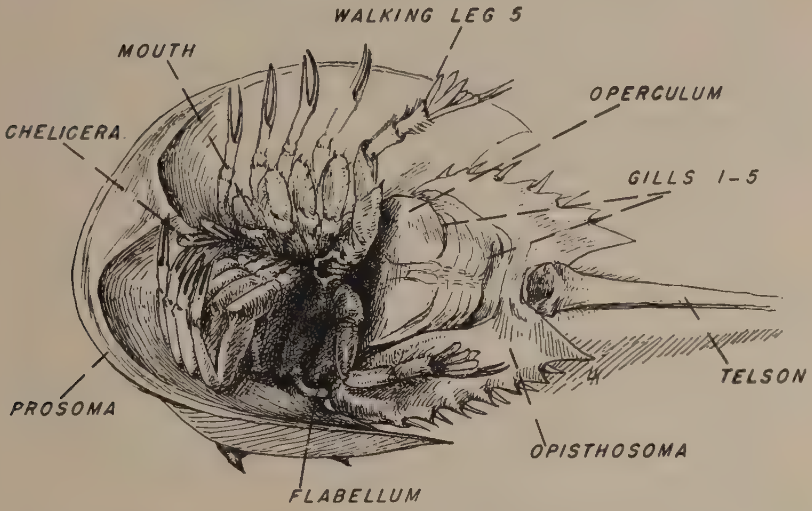

Fig. 1 Ventro-lateral aspect of an immature *Limulus* (50 mm prosoma length). The legs on the nearer side have been displaced to show the position of the flabellum as an epipodite of the 5th pair. Its relation to the incoming respiratory channel at the antero-lateral gill margins is also clear. Drawn by Lisbeth Krause.

Respiratory reflexes

The critical factors in breathing are two: the mass of air or water moved over the respiratory surface per unit time and the efficiency of transfer of carbon dioxide and oxygen across this boundary between the medium and the body. Only the former factor has been studied in the present work. This was done indirectly by two different means in experiments testing the effects of various partial pressures of respiratory gases.

First, kymographic records of gill-book activity were made. The frequency and amplitude of these movements provide a good quantitative estimate of water convection over the respiratory surfaces. Second, a parallel series of data were obtained on the frequency of gill beats in free animals unencumbered with recording attachments. Although clearly a less adequate measure of ventilation volume, these latter experiments were desirable for purposes of comparison. Previous workers have found quite different results in decapod crustaceans depending on whether or not the animal was fastened down and the scaphognathite attached to a recording lever (von Buddenbrock, '48; Harnisch, '51, p. 152). Consequently, an effective experimental design must include adequate control of this matter.

MATERIAL AND METHODS

Immature specimens of *Limulus polyphemus* (L.) about 50–100 mm in prosoma length were used in these experiments. For kymographic recording, the animals were fastened with the ventral side up. A fine hook connected by a thread to a light isotonic recording lever was inserted in the distal margin of the second or third gill. In this condition the animal was placed on the bottom of a closely covered vessel of seawater, whose gas content was altered by bubbling in various gas mixtures through porous aerating stones. Since the capacity of the vessel was 14 l, a period of some minutes was required to reach a steady state when gas mixtures were changed. Despite this, however, the results showed that the animal's responses were immediate to certain of such changes.

For the data on the free *Limulus* the gas content of the seawater was already in a steady state when the animals were immersed. Changes in this gas content were effected by moving the animals from one container to another. It was found that reliable measurements of gill beat frequency could be made under these conditions if great care was exercised in using a standardized transfer procedure and a randomized order of exposures. An exception was the case of high partial pressures of carbon dioxide which even in a short time exert such relatively long lasting general depressant action that this interacts to mask the more immediate reflexes involved in ventilation.

The specific measurement made in this other set of experiments was the time in seconds (measured with a stop watch) required for the horseshoe crab to make 25 gill beats after being immersed in seawater saturated with a given gas mixture. Gill beats were counted with the animal in its normal orientation on a substrate by conducting the experiments in glass vessels raised far enough for a mirror to provide a clear view of the ventral surface.

Gas analysis of the seawater at various times during the experiments was made for oxygen and for carbon dioxide. As the size of the water samples was not critical, standard Winkler procedure (Harvey, '45) was used for the former determination. Total CO_2 was measured by an evolution method essentially like that described in Standard Methods for Examination of Water and Sewage (Am. Publ. Health Assoc. and Am. Waterworks Assoc., '49, p. 32). It should be noted that the absence of calcification in the *Limulus* exoskeleton (Lafon, '43) obviated the complex interactions found with the $CaCO_3$ in shells of bivalves (Galtsoff and Whipple, '31) and integument of crustaceans (Peters, '38). Determinations of pH were made electrometrically and colorimetrically.

RESULTS

The normal breathing movements of *Limulus* consist of alternate adduction and abduction of the 5 fused pairs of overlapping gill-bearing appendages (the second through 6th abdominal pairs). Each of these bears on the proximal lateral parts of its posterior surface two gill books. The first pair of adult abdominal appendages are fused to form the genital operculum, which covers the gills when adducted, and in general, beats in rhythm with them in eupnea.

Three sets of muscles: abductors, adductors and the intersegmental branchiothoracics (Patten and Redenbaugh, 1899), are the effectors driving this system. Respiratory centers in the abdominal ganglia and in the posterior neuromeres of the subesophageal components of the cephalothoracic nerve-ring are known from transection experiments to be involved in its control (Hyde, 1894, '06; Patten, '12, pp. 174 ff.).

The hydraulics of the gills' rhythmic movement is such that a periodic current of water is drawn into the gill books from the anterolateral corners of the opisthosoma and discharged medially along the distal margins of the gills. Presumably, then, water is sucked in laterally between the 100 or more "pages" of the gill books by abductors which tend to open out these lamellae. Adduction squeezes them shut and jets the water posteroventrally. Although the details have not yet been studied, a sort of double metachronal rhythm seems to be present in the gill movements. In abduction the operculum starts first followed in turn by each more posterior pair of appendages, the inhalent current thus being drawn successively over the gill books from fore to aft. The exhalent phase, on the other hand, starts posteriorly with the adduction of the 5th gills succeeded by the more anterior gills and the operculum seriatim.

The frequency of gill beats in animals of the size used in these experiments when they were immersed in air-saturated seawater at 22°C. averaged about 35 per minute. However, different individuals and even the same individual at different times showed marked variations (the overall range extended from 20–50 beats per minute).

The normal steady beat of the gills (as seen at the beginning of fig. 2 D) may be modified by several factors. In the restrained animal, ventral side up, struggling movements of the legs or attempted flexion of the opisthosoma may interfere with the regular pattern. Swimming movements (fig. 3 A) in which the appendages of both prosoma and opisthosoma participate in metachronal beats, interrupt eupneic breathing although they do, nonetheless, provide adequate gill ventilation thereby. Also "cross-cleaning" activity of the gills themselves stops normal breathing. Here the medial distal tabs of one member of the pair slide over and "inter leaf" the gill book of the opposite side. A 4th variation on the continual steady ventilation pattern was observed on several occasions in the form of intermittent breathing like Cheyne-Stokes respiration in man. Such discontinuous ventilation patterns are probably widespread in animals as they have already been reported for annelids, crustaceans, molluscs, insects, fishes, amphibians, reptiles and mammals (for discussion see van Dam, '38, pp. 132 ff.).

Obviously, the experimental effects of various gas mixtures have to be determined and interpreted with this basic normal pattern of respiratory activity and its modifications in mind.

When the oxygen content of the seawater was reduced, a characteristic series of changes in respiratory movements ordinarily occurred. In the inverted animals, with one of the gills recording its beats kymographically, the following took place. As soon as nitrogen was bubbled into the medium, the O_2 content of the latter began to fall from its normal air saturated content of 5–6 ml per liter to less than 0.5 ml per liter in 15–30 minutes. Meanwhile, the amplitude and frequency of the gill beats steadily declined. In the example shown in figure 2 A, 24 minutes after the N_2 began to be introduced, the amplitude had dropped to 50% of its initial value, frequency from 47 per minute to 20 per minute. Twenty-three minutes later, amplitude was only 25–30% its original size and beat frequency was 16 per minute, about 30% its value in air-saturated seawater. Five minutes later, breathing had stopped completely. The operculum was partly closed in this final stage.

More or less complete ''systolic'' stoppage of the gills was characteristic of *Limulus* in anoxia under such conditions of recording. In a total of 10 trials on 9 different specimens: 5 showed complete systolic closure of the operculum within 27 minutes, two shut partially when breathing stopped, the remaining three, although showing anoxic stoppage, did not tend to close the gills. Since the present interest in these respiratory reflexes centered on possible immediate sensory involvement, no long term experiments were made to study the duration of closure, period of anoxic survival and the like.

Now, if O_2 was bubbled into the seawater of a container with the horseshoe crab whose gills were in anoxic arrest, an immediate response was obtained. This was found in all cases, whether or not the operculum and gills were tightly closed. Within 5 seconds, breathing movements started and immediately became normal, or temporarily supranormal, in both amplitude and frequency (fig. 2). The first two or three gill movements were relatively slow and of small amplitude, but succeeding ones increased rapidly in both of these dimensions as the records attest. Note that the rapidity of this oxygen response precludes the prior attainment of a steady-state condition of the gases dissolved in the large experimental container.

In order to be certain that this was an immediate response to oxygen *per se* other gases were tried under similar conditions. If the N_2 was turned off for one minute while the gills were in systolic stoppage, an interesting result was obtained in a number of tests when the same gas was turned on again (fig. 2). In this case the operculum would open somewhat and slow gill beats of low amplitude begin as in the case of O_2. But with N_2 they remained relatively slow

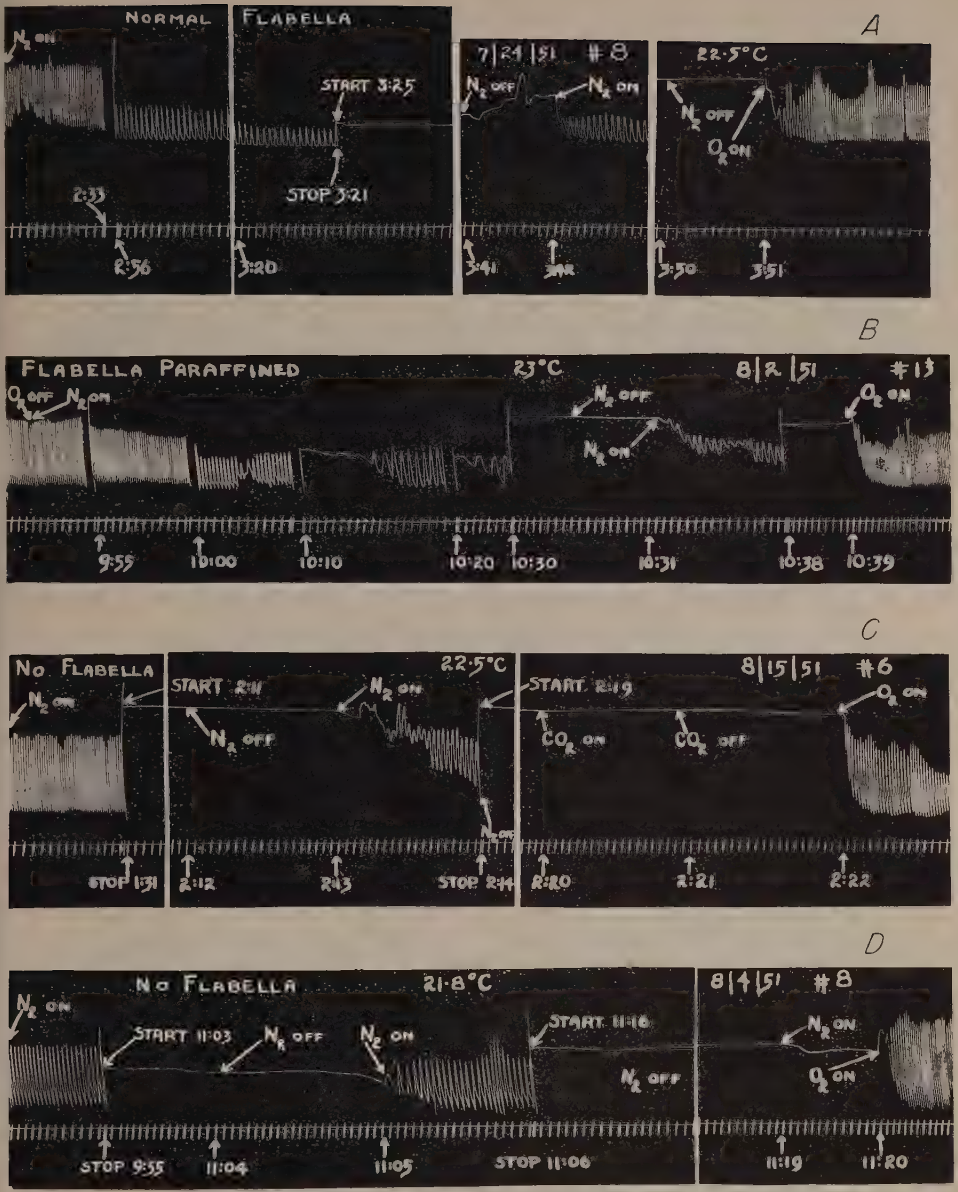

Fig. 2 Kymograph records illustrating effects of O_2 and O_2-lack in seawater on *Limulus* gill movements. Time marks at 5 second intervals except as indicated. In *A*, the reactions were those of an animal with intact flabella; in *B*, these organs had been completely covered with paraffin wax and in *C* and *D*, they had been previously amputated at the basal articulation. In all cases note that anoxic anoxia developed without hyperventilation, that O_2 instantly evoked breathing during anoxic arrest, and that O_2, N_2 and CO_2 could be immediately discriminated by the animal. Comparison of the data gives no indication that flabellar chemoreceptors were involved in these reflexes.

and small. Shortly the gills would stop again in systolic arrest. If CO_2 was tested in the same way O_2 and N_2 were above, no effect of short exposures was observed on the respiratory system when it was in systolic stoppage (fig. 2 C).

Carbon dioxide did, however, have marked effects on eupneic breathing (fig. 3). Immediate disruption of normal beat followed the introduction of CO_2 into the vessel with the *Limulus* recording its gill movements. As will be seen below, this does not happen in a free animal walking around untrammeled with recording gear. In the kymographic records, the base line begins to rise at once, large irregular beats may occur, while the regular breathing pattern decreases in amplitude and frequency, or may be replaced by the cross-cleaning movements described above. In a few minutes, the gill beats have disappeared completely (fig. 3 A) or been reduced to negligible amplitude (fig. 3 B). Finally, ventilation arrest occurs in "diastole" i.e., with the operculum and gills gaping open. Complete diastolic stoppage appeared in 16.5 minutes on an average for 12 out of a total of 14 experiments on 11 different animals.

By this time, the total CO_2 content of the seawater had risen from a normal content for air saturation of about 50 ml per liter to over 200 ml per liter. At the same time, the pH of the medium dropped from normal of about 8 to pH 5.2. Concomitant decrease in the O_2 content of the water also occurred under the conditions of the experiment. As with N_2, introduction of CO_2 reduces the normal value of O_2 for air saturation to less than 0.5 ml per liter. For present pur-

Fig. 3 Kymograph records illustrating the effects of CO_2 and O_2 in seawater on *Limulus* gill movements. Time marks at 5 second intervals except as indicated. In *A*, the reactions were those of an animal with flabella intact; in *B*, these organs had been previously amputated at the basal articulation. Observe that in CO_2 anoxia breathing stoppage occurs in the "diastolic" phase of the gill beats compared with the systolic stoppage in anoxic anoxia (fig. 2). The records show that both CO_2 and O_2 produced prompt changes in respiratory pattern and that O_2 was distinguished from N_2 and CO_2. No indication is given implying the involvement of flabellar chemoreceptors in these responses.

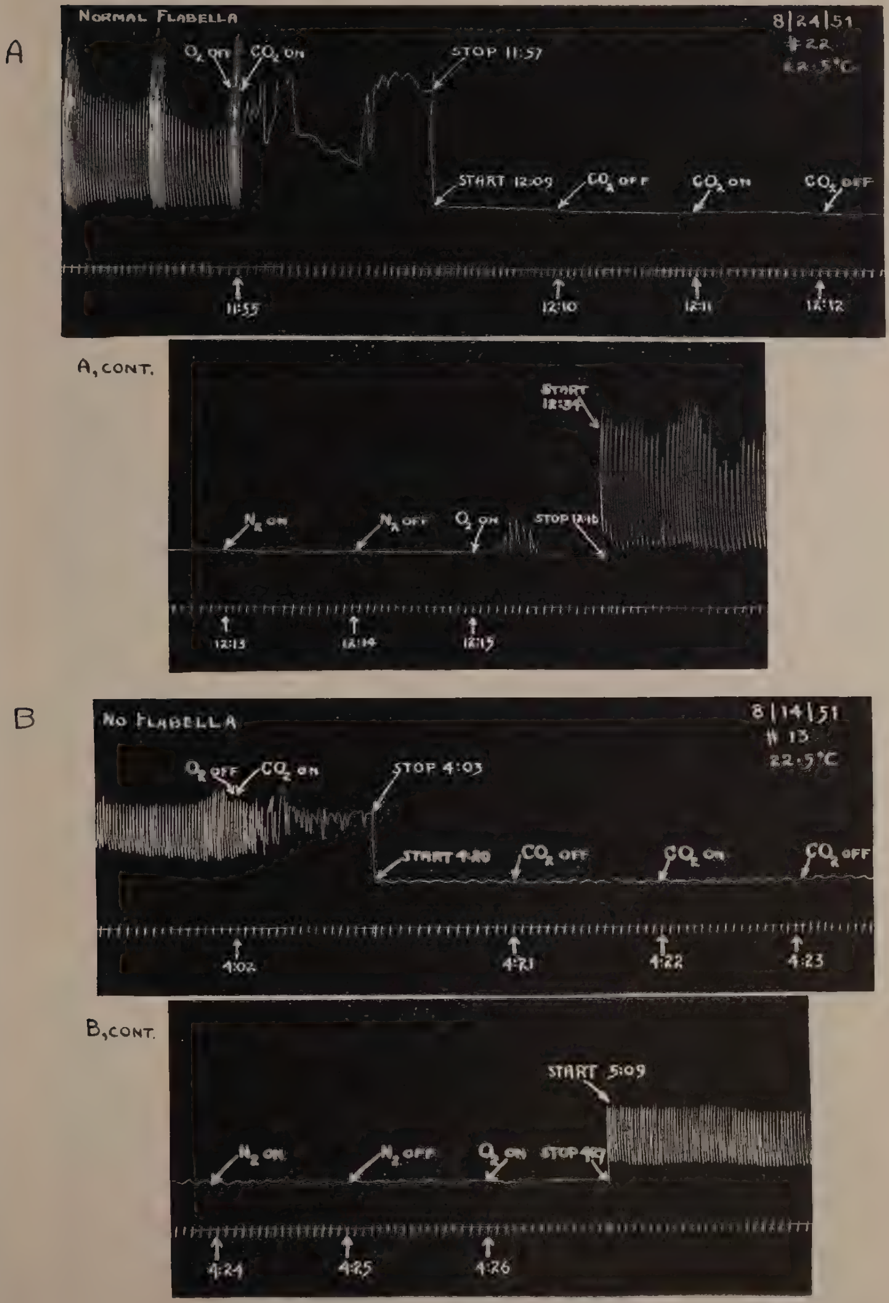
A
NORMAL FLABELLA
8|24|51
# 22
22.5°C
O_2 OFF
CO_2 ON
STOP 11:57
START 12:09
CO_2 OFF
CO_2 ON
CO_2 OFF
11:55
12:10
12:11
12:12
A, CONT.
START 12:34
N_2 ON
N_2 OFF
O_2 ON
STOP 12:16
12:13
12:14
12:15
B
NO FLABELLA
8|14|51
# 13
22.5°C
O_2 OFF
CO_2 ON
STOP 4:03
START 4:20
CO_2 OFF
CO_2 ON
CO_2 OFF
4:02
4:21
4:22
4:23
B, CONT.
START 5:09
N_2 ON
N_2 OFF
O_2 ON
STOP
4:24
4:25
4:26

Figure 3

poses no attempt was made to separate these pH and O_2 interactions with the specific effect of CO_2 itself.

Specific restorative action of O_2 on the respiratory beat of *Limulus* in CO_2 arrest was demonstrated in all cases. Recovery was not complete in one case and was slow in others. However, for half the tests some ventilation was restored in less than a minute while in the remainder, one minute or more was required for this response to appear. Again, experiments were run to determine whether this was a specific O_2 effect. Clearly, it was, since neither CO_2, turned on and off, nor N_2 had any immediate effect on the diastolic stoppage (fig. 3).

The control data on free, unencumbered *Limulus* showed that the initial rate of breathing on exposure to a particular gas mixture varied directly with the O_2 content of the seawater (fig. 4). In other words, the results obtained kymographically, where the frequency of ventilation movements decreased steadily as the O_2 content of the medium diminished (fig. 2 A), were in this important respect the same as those of animals where neither normal orientation nor movements were interfered with. The results thereby differ from those, cited above, with some of the decapod crustaceans where quite different responses have been found under the two comparable conditions of experiment.

Summary of the results of 450 trials on 5 animals shows that most rapid gill beats occurred in O_2-saturated seawater, least rapid in an N_2-saturated medium, while air-saturated water evoked an intermediate frequency. Despite the small differences in the mean rates of breathing observed, analysis of variance in the original data shows that these differences are highly significant, i.e. F values for the gas mixtures exceed the tabular values at the 1% level.

These particular experiments do not include data on CO_2-saturated seawater, because even a short exposure to this affected *Limulus* so profoundly that strong interactions occurred in any subsequent tests. Some data were collected, however, on the effect of saturating the medium with 95%

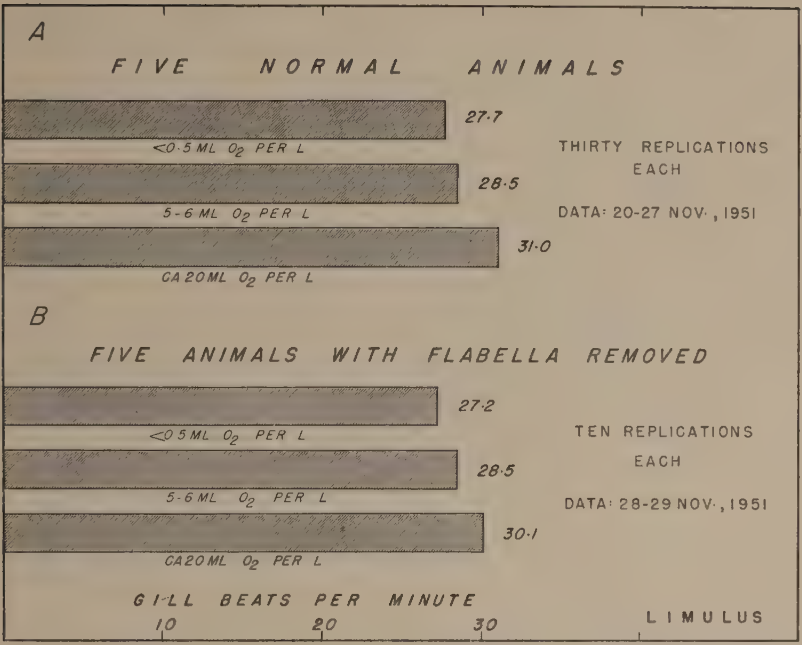

Fig. 4 Frequency of gill beat in *Limulus* exposed to various concentrations of O_2 in seawater. Data represent breathing rate for the first 25 beats after the experimental animal was transferred from one O_2 concentration to another. Three important points are illustrated. (1) The animal detected and responded to the variation in O_2 content of the medium since small but statistically significant differences in breathing rate occurred. (2) The gill beat frequency varied directly with the O_2 concentration, and at least these initial responses showed no signs of dyspnea. (3) The data contraindicate the involvement of chemoreceptors of the flabella in these responses.

O_2, 5% CO_2. These cannot be plotted together with the results shown in figure 4 since they were from another series of measurements, but they show clearly that in seawater saturated with 95% O_2, 5% CO_2, gill beats are considerably slower even than in N_2-saturated O_2-free medium. This suggests an immediate respiratory reflex sensitive to CO_2 (or pH) which should be further investigated.

DISCUSSION

The above results on the ventilation of *Limulus* exposed to various concentrations of respiratory gases are of interest in two particular respects. First, the data are noteworthy because they show clearly that external chemoreceptors must be involved in certain of the respiratory reflexes. Most striking was the immediate response to O_2 shown when breathing had stopped in anoxic arrest. This was so rapid (within 5 seconds) that equilibrium was far from being achieved in the experimental vessel, let alone in the blood stream of the animal. Under these same conditions the animal could readily distinguish between O_2, N_2 and CO_2 (figs. 2, 3). In addition, suggestive evidence is contained in the data of figure 4 which implicates peripheral exteroceptors in establishing the frequency of gill movement. Here again the response to changes was so immediate that interoceptors seem unlikely.

Such results fit in with those of Hyde ('06) demonstrating that afferent impulses were essential for maintaining the rhythmic activity of a *Limulus* branchial ganglion. Her work needs repeating, however, since the branchiothoracic musculature and its innervation were ignored at the time (Patten, '12). Modern electrophysiological techniques also would permit more incisive analysis of the problem. In any case, the present experiments add another definite example to the ones cited above where external partial pressures of respiratory gases have been proved to be perceptible to various animals. Further study will undoubtedly augment instances of this sort.

The second interesting point about the present experimental data is that they indicate no sign of dyspnea at the onset of respiratory emergencies in the environment. In fact, with the animals recording gill movements kymographically, no period of hyperventilation appeared at all during the 30–60 minutes of exposure to low O_2 or high CO_2 partial pressures. This is consonant with previous observations that *Limulus* shows no regulation of O_2 consumption over wide ranges of environmental O_2 partial pressures (Amberson, Mayerson and Scott, '24; Maloeuf, '37). But O_2 consumption itself depends on so many other complex functions that a number of parameters must be studied to find its significance.

In the present experiments supranormal amplitude or frequency of gill beats was recorded at the end of an exposure to anoxic conditions in 50% of the trials. Yet this lasted only for one or two minutes even when the preceding periods in seawater with less than 0.5 ml per liter lasted 30–60 minutes. Obviously, experiments of longer duration, particularly with free-moving *Limulus,* would be required to give further insight into possibilities of anaerobic processes and oxygen debt. Also, more information on the *in vivo* activities of hemocyanin, the respiratory function of *Limulus* blood, and the underlying tissue metabolism of the animal are needed to analyze the whole picture adequately.

There are some indications that physiological chemical patterns rather different from those found in more thoroughly studied organisms are present here. For example, *Limulus* oxyhemocyanin apparently has its copper oxidized to the cupric state while reduced hemocyanin's copper is cuprous (Schulman and Wald, '51). This stands in sharp contrast to the oxygenation and deoxygenation of a respiratory pigment like vertebrate hemoglobin. Furthermore, the Bohr effect was found to be reversed in the horseshoe crab's hemocyanin dissociation curves (Redfield, Coolidge and Hurd, '26), a fact difficult to reconcile with effective respiratory transport by this copper-protein. And the actual position, relative to partial pressures of O_2, of the O_2 dissociation curve likewise makes the importance of this hemocyanin's respiratory transport questionable (Maloeuf, '37). It is curious that so little is known about the actual functioning of hemocyanin in *Limulus* when much of the classic work on the *in vitro* biochemistry of the pigment has been done in this animal (Redfield, '34; Wolvekamp, '49).

The CO_2 transfer mechanisms also seem unusual since large amounts of carbonic anhydrase have been found in the horseshoe crab but little occurred in the blood (Ferguson, Lewis and Smith, '37). Amounts comparable to those present in vertebrate blood were localized in the gills and appreciable quantities in muscle tissue. These facts imply quite unfamiliar relationships.

The absence of dyspnea in *Limulus* exposed to respiratory stress may seem a peculiar finding since compensatory respiratory and circulatory responses to such conditions are so common and widespread in animals. However, there are a number of interesting precedents to be found. In the crayfish, *Astacus* for example, with a restrained animal and with recording levers attached to the scaphognathites, the appendages that drive the respiratory currents, both low O_2 and high CO_2 inhibited ventilation (Segaar, '34). In free, unrestrained animals, on the other hand, these conditions have generally been found to induce dyspnea and not inhibition (Peters, '38). But other observers found temporary apnea at the beginning of an exposure to high CO_2 concentrations (Jordan and Guittart, '38). A similar inhibition was described for the crab, *Eriocheir,* in which moderate pressures of CO_2 induced a diphasic change in the breathing pattern, an immediate inhibition followed by hyperventilation (van Heerdt and Krijgsman, '39). In the vertebrates, CO_2-sensitive reflexes are known to inhibit breathing in certain species of ducks (Hiestand and Randall, '41). Even less conventional is the situation found in certain insect larvae which normally live in an environment high in CO_2. Not only are the usual reflex opening of the spiracles and hyperventilation on exposure to high partial pressures of this gas lacking, but these larvae may actually die in its absence (Levenbook, '51).

A suggestive situation occurs in a tubicolous aquatic insect larva *(Chironomus plumosus)* which irrigates its burrow for feeding and respiration. This animal normally shows only dyspneic behavior under anoxic conditions (Walshe, '51). Its ventilation periods, intermittent and brief in air-saturated water, lengthen in decreasing O_2 tensions until at 5% air saturation, ventilation is continuous. This fresh water form then ordinarily shows a respiratory pattern of the conventional type. If, however, its respiratory pigment, hemoglobin, is inactivated with carbon monoxide, hyperventilation does not occur under anoxic conditions. Instead, lowered O_2 tensions here result just in decreased ventilation, as they do in normal *Limulus*.

A more general interesting parallel to the kinds of respiratory responses found in *Limulus* may be seen in sedentary intertidal marine animals. Here the response pattern takes on a clearly adaptive significance. As a rule, sessile forms like sponges, anemones, corals, molluscs and barnacles become inactive during ebbtide. Obviously, this happens when the animals are exposed to the air but may also when unfavorable conditions develop in small isolated tide pools. Thus, Parker ('10) found that the marine sponge *Stylatella* shut its osculum in deoxygenated seawater. Comparable behavior has been described in other sponges. Actinians closed up when confined under anoxic conditions as did the gastropod *Littorina* (Piéron, '08). In these latter cases at least, the response definitely postponed asphyxia. Oysters, also, showed increasing periods of shell closure at experimentally reduced O_2 tensions and finally shut continuously when the O_2 fell below 1–1.5 ml per liter (Nozawa, '29).

Similar responses occurred in tube-living polychaete worms like *Arenicola* and *Sabella*. In these and other intertidal annelids ventilation of the burrow has been found to take place in a spontaneous intermittent pattern. If aeration of the water irrigating the tubes was prevented (as it often is at low tide), the ventilating movements of the worm stopped for long periods (up to 8.5 hours). On aeration of the water again irrigation, now supranormal, was immediately resumed (Wells, '51).

Observe that despite its apparent eccentricity, the kind of breathing stoppage here described is widespread in the animal kingdom, occurring already in the simplest of multicellular animals. The prevalence of such a function, arising in many groups independently by phylogenetic convergence, attests to its broad significance.

Thus, further work may show the observed respiratory reflexes of *Limulus* to be ecological adaptations of this sort. Periodically adverse respiratory conditions would arise during the seasons when horseshoe crabs occur intertidally. The almost certain early tidal return of favorable respiratory circumstances would permit them to conserve their energy by respiratory shutdown, fatal to organisms in other environments where the animal's own positive responses are the only likely means of obviating the unfavorable conditions. A rough analogy to this respiratory behavior may be seen in the divergent ways terrestrial animals meet seasonal climatic stress either by the metabolic abatement which underlies hibernation or by a more intensive response like migration (Prosser, '50, p. 371).

The flabellum

Inter alia the foregoing data on *Limulus* respiratory reflexes implicated peripheral chemoreceptors somewhere on the surface of the body. Specifically, the ability to detect O_2, instantly it was added to water of low O_2 content, and the immediate correlation of breathing frequency with O_2 and CO_2 tension of the seawater definitely seemed to require exteroceptors sensitive to these gases. As mentioned above, Hyde ('06) also had obtained suggestive evidence that such sense organs must be present. She pointed out among other things that since breathing continues in bloodless animals for as long as 40 hours, regulation of gill beat rhythm could not be reflexly dependent on the respiratory state of the blood, detected by interoceptors, as it is in the higher vertebrates. Yet her work indicated that the ventilation rhythm in the branchial ganglia of *Limulus* was reflex in origin and was influenced by a remarkable variety of afferent nervous connections with other parts of the body, including the viscera. This section of the present paper deals with a study of the flabellum as a possible site of these respiratory exteroceptors.

Morphology and histology.[3] The flabella of *Limulus* are prominent organs of unknown function articulating, as epipodites, on the coxae of the 5th pair of walking legs (fig. 1). Each consists of an unjointed paddle-shaped structure with a proximal section, ovoid in cross-section, and a spatulate distal moiety, thin and blade-like. The flabellar axis, which is approximately horizontal, is bent backward at the level of the distal end of the basal region so that the blade is directed posteriorly. The latter lies, then, as a dorsoventral lamella in that part of the branchial region bathed by the incoming respiratory current.

On its exposed outer surface, the blade of the flabellum is provided with many thousand microscopic pores. Two sizes occur, but the smaller ones, by far the most numerous, are restricted just to this flat anterolateral flabellar surface. The following details will refer to this specific type of pore. Branches of the large flabellar nerve innervate these units (figs. 5, 6) and the pores appear to be the external, cuticular component of a highly developed sense organ (figs. 7, 8).

Starting peripherally, these structures are constituted as follows. The external pore is a cup-like depression in the exoskeleton about 8 μ in diameter.[4] Distance between pores is 15–20 μ. At the bottom of each pore-cup is a smaller aperture or thin plate 1–2 μ in diameter. This marks the outer end of a tubule (40 μ or more long) which runs from this point through the cuticle and epidermis in toward a cluster of nerve cell bodies. At its inner end this tubule, which stains histologically like the epicuticle, becomes less distinct as it approaches a structure resembling the scolopalia of insect and crustacean sensilla. This scolopale contains a number of rod-like elements and apparently terminates in an apical filament. At least in the center of the epicuticle covering the end of the tubule, a fine spot about 0.25 μ in diameter may be seen; this could be the point of terminal attachment of such a filament although one has not been seen with certainty in sections cut parallel to the tubule. Patten ('12, p. 114), in the only previous work on these organs, did see such an apical filament.

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[3] The authors are indebted to Mrs. Margaret W. Abel and Miss Anne Cobey for assistance in preparing the histological material used in this work.

[4] The figures given indicate the order of magnitude for the elements concerned in specimens about 50 mm in prosoma length.

Proximally, the neck of the scolopale leads into a subcylindrical or pear-shaped mass of cells whose precise relations have not been made out from the present work. The whole structure is supported by a tenuous connective tissue sheath and each cluster receives the canals originating in a number of the cuticular pores (figs. 7, 8). From the inner end of this cell mass emerges a bundle of sensory nerve fibers which join the flabellar nerve (figs. 5, 6). Sheath cells are present around the outer parts of these structures where the canals run to the epidermis, through it, and to the outer end of the cuticular channel.

The close parallel of these structures in *Limulus* to certain other sensilla of arthropods is striking. Particularly, their resemblance to structures believed to be chemoreceptors in insects, crustaceans, arachnids and elsewhere in *Limulus* cannot be ignored. However, few are the instances where direct positive correlation of chemoreceptor function and specific sensilla type has been possible (Dethier and Chadwick, '48; Frings and Frings, '49; Roth and Willis, '51). In any case, such sense organs have been quite refractory to electrophysiological analysis, a most critical datum for their quantitative study. Recently, a promising beginning in this direction has been made with the rather similar contact chemoreceptors on the circumoral gnathobases of *Limulus* walking legs (Barber, '51).

During the current work, a few exploratory studies of action potentials in the flabellar nerve have been carried out. As yet, they have not produced any chemical responses of significant nature. Further research along these lines is crucial to understanding the significance of the flabellum.

Experiments on the relation of the flabella to breathing. The foregoing data have shown clearly that respiratory reflexes in *Limulus* were initiated in part by external chemoreceptors. Furthermore, the flabellum was found to be the animal's largest sense organ which from its structure might be an exteroceptor stimulated chemically. To discover whether or not there was any physiological correlation between these facts, the following experiments were tried.

The whole outer surfaces of the flabella, where the sensory pores were located, either were covered with paraffin wax or the appendages themselves were amputated completely. Both kymographic recordings and simple gill beat frequency measurements were carried out under these two conditions. The techniques and procedures used were the same as those described for normal animals in the first part of this report. Comparison of results obtained with the flabella blocked or removed, and the normal respiratory reflexes would thus indicate how far these organs were involved in such responses.

Reference to figures 2 B, 2 C, 2 D and 3 B will demonstrate that no significant changes in breathing resulted either from covering the special flabellar sense organs or entirely removing the structures. Respiratory patterns in normal air-saturated seawater and the various alterations in gill beat produced by different gases in the water were quite unchanged. Similarly, the rhythm or initial frequency of breathing with various respiratory gas mixtures was not altered by bilateral flabellum removal (fig. 4 B). Detailed comparisons of these two sets of experiments will be evident from figures 2, 3 and 4.

CONCLUSIONS

The experimental data presented above have demonstrated the presence in *Limulus* of several reflex responses to short term changes in external O_2 and CO_2 tension. Like the early results of Hyde and Patten, those reported here indicate that sense organs on the external surface of the body were concerned in establishing the frequency of gill beat and also in initiating changes in the breathing pattern. In addition, the present data provide several definite respiratory reflexes which can now be utilized quantitatively in searching for and analyzing the respiratory chemoreceptors. Most promising of these reflexes was the immediate response of the gills to O_2 when they were in a state of systolic anoxic arrest. The rapid effect of increased CO_2 on normal breathing under similar conditions of kymographic recording would also offer a useful test. Thus far, only the apparent chemoreceptors of the flabellum have been tested for effects on such respiratory activity. The negative results obtained here showed that even if the sensilla involved were sensitive to any of the environmental changes tested, the responses did not have a determining effect either on the respiratory rhythm or on the respiratory reflexes.

To pursue this matter further, other chemoreceptors on the body surface of *Limulus* need to be examined. The most promising structures to try are the blister-like units that lie on the anteroventral surface of each gill's distal end. These were shown by Patten ('12, p. 114) to be perforated by numerous remarkable bell-shaped pores, connected to well innervated sensilla. Furthermore, the gill-books themselves receive an appreciable sensory innervation (Patten and Redenbaugh, 1899) which could be involved in such reflexes.

In addition, the application of electrophysiological techniques to the problem of *Limulus* respiration as a whole should be most rewarding. Not merely would this help solve the chemoreceptor problem, but it could shed much light on the complex integration of the whole breathing pattern suggested by the operative experiments of Hyde and Patten. Many aspects of the question directly insoluble by other methods would yield to this approach.

From a more biochemical point of view, data of the sort described here should be extended so that they can be correlated with the problems of respiratory transport by the animal's circulatory system and with the possibilities of anaerobic respiration by its tissues. *In vivo* studies of hemocyanin function and of metabolic pathways in *Limulus'* tissues would be pertinent here.

Finally, the ecological implications of the horseshoe crab's respiratory reflexes should be explored further. It would be most interesting to establish whether the animal's lack of hyperventilation in the face of respiratory stress is significantly correlated with its seasonal occurrence in the intertidal zone, a habitat which could make such behavior of adaptive value.

SUMMARY

1. This report describes experiments designed to demonstrate the nature of short term respiratory regulation in the horseshoe crab, *Limulus polyphemus* and to test the hypothesis that the flabellum is an external chemoreceptor involved in such reflexes.

2. Ventilation was gauged by two methods, kymographic recording of the amplitude and frequency of breathing movements in restrained animals and just measuring the frequency of gill beat in free animals exposed to various concentrations of the respiratory gases.

3. Seawater content of respiratory gases was modified by aerating with O_2, N_2 or CO_2 gases. Total CO_2 content, O_2 content and pH were quantified at critical points during the experiments.

4. Normal respiratory movements consist of alternate adduction and abduction of the fused pairs of abdominal appendages at a frequency of about 35 per minute with *Limulus* of 50 mm prosoma length in air-saturated seawater.

5. Anoxia led to a steady decline in frequency and amplitude of gill movements in the attached animals finally resulting in complete arrest after about 30 minutes, with the gills more or less tightly closed (fig. 2). In the free animals, too, frequency of breathing varied directly although to a small degree, with the partial pressure of O_2 (fig. 4). No hyperventilation occurred in either group of animals under the stress of anoxia.

6. Evidence is marshalled that respiratory behavior of this sort is a widespread adaptation to certain environ-

mental conditions in many animals and may prove to be so in *Limulus.*

7. Excess CO_2 produced immediate disruption of the normal respiratory pattern in attached animals and in about 15 minutes led to complete stoppage of breathing with the gills gaping (fig. 3). In free animals similar conditions led to a slowing of gill beats followed by anesthesia. Dyspnea did not occur in either case.

8. Respiratory arrest, whether produced by anoxia or excess CO_2, was rapidly terminated when the seawater was aerated with O_2. This response, as well as certain other reflexes evident in figures 2, 3 and 4, proved that chemoreceptors on the external body surface must be involved. These exteroceptors were able to distinguish O_2, N_2 and CO_2 from one another.

9. Since the flabella (fig. 1) contain the largest sense organs which might be chemoreceptors on the animal's body surface, their histology was studied (figs. 5, 6, 7 and 8), and their effect on the foregoing respiratory reflexes was tested.

10. Experiments repeating the various exposures to different respiratory gas concentrations showed clearly that the same responses obtained in normal animals occurred when the flabellar organs were covered with wax or the whole structure amputated (figs. 2, 3 and 4). Therefore, these sense organs are not the respiratory exteroceptors involved in the reflexes described above.

LITERATURE CITED

AMBERSON, W. R., H. S. MAYERSON AND W. J. SCOTT 1924 The influence of oxygen tension upon metabolic rate in invertebrates. J. Gen. Physiol., *7:* 171–176.

AMERICAN PUBLIC HEALTH ASSOCIATION AND AMERICAN WATERWORKS ASSOCIATION 1946 Standard Methods for Examination of Water and Sewage. Ed. 9. Am. Publ. Health Assoc., New York. Pp. 1–286.

BARBER, S. B. 1951 Contact chemoreception in *Limulus.* Anat. Rec., *111:* 561–562 (abstract).

v. BUDDENBROCK, W. 1948 Physiologie der Decapoden. Bronn's Klassen und Ordnungen des Tier-Reiches. Bd. 5, Abt. 1, Bch. 7, Lief. 8. Pp. 1108–1137.

v. Dam, L. 1938 On the utilization of oxygen and regulation of breathing in some aquatic animals. Dissert. Groningen. Drukkerij Volharding, Groningen. Pp. 1–143.

Dethier, V. G., and L. E. Chadwick 1948 Chemoreception in insects. Physiol. Rev., *28:* 220–254.

Ferguson, J. K. W., L. Lewis and J. Smith 1937 The distribution of carbonic anhydrase in certain marine invertebrates. J. Cell. and Comp. Physiol., *10:* 395–400.

Frings, H., and M. Frings 1949 The loci of contact chemoreceptors in insects. Am. Midl. Nat., *41:* 602–658.

Galtsoff, P. S., and D. V. Whipple 1931 Oxygen consumption of normal and green oysters. Bull. U. S. Bur. Fish., *46* ('30): 489–508.

Gronovius, L. T. 1764 Zoophylacium Gronovianum. Lugduni Batavorum. Vol. 2.

Harnisch, O. 1951 Hydrophysiologie der Tiere, Elemente zu ihrem Aufbau. Die Binnengewässer, *19:* 1–299.

Harvey, H. W. 1945 Recent Advances in the Chemistry and Biology of Sea Water. University Press, Cambridge. Pp. 1–164.

v. Heerdt, P. V., and B. J. Krijgsman 1939 Die Regulierung der Atmung bei *Eriocheir sinensis* Milne Edwards. Zschr. vergl. Physiol., *27:* 29–40.

Heymans, C., and P. Rijlant 1933 Le courant d'action du nerf du sinus carotidien intact. C. rend. Soc. Biol., *113:* 69–73.

Hiestand, W. A., and W. C. Randall 1941 Species differentiation in the respiration of birds following carbon dioxide administration and the location of inhibitory receptors in the upper respiratory tract. J. Cell. and Comp. Physiol., *17:* 333–340.

Hyde, I. H. 1894 The nervous mechanism of the respiratory movements in *Limulus polyphemus.* J. Morph., *9:* 431–448.

——————— 1906 A reflex respiratory centre. Am. J. Physiol., *16:* 368–377.

Jones, R. M. (Editor) 1950 McClung's Handbook of Microscopical Technique. 3rd Edition. Hoeber, New York. Pp. 1–790.

Jordan, H. J., and J. Guittart 1938 Die Regulierung der Atmung bei *Astacus fluviatilis.* Proc. Kon. Nederl. Akad. v. Wetensch., Amsterdam, *41:* 2–9.

Lafon, M. 1943 Sur la structure et composition chimique du tegument de la Limule *(Xiphosura polyphemus* L.). Bull. Inst. Oceanogr., No. 850: 1–11.

Levenbook, L. 1951 The effect of carbon dioxide and certain respiratory inhibitors on the respiration of larvae of the horse bot fly *(Gastrophilus intestinalis* de Geer). J. Exp. Biol., Lond., *28:* 181–202.

Linnaeus, C. 1758 Systema Naturae. 10th Edition. L. Salvii, Holmiae. Tome 1: 1–824.

Maloeuf, N. S. R. 1937 Studies on the respiration of animals. II. Aquatic animals with an oxygen transporter in their internal medium. Zschr. vergl. Physiol., *25:* 29–46.

MÜLLER, O. F. 1785 Entomostraca seu Insecta Testacea. Mülleriani, Lipsiae et Havniae. Pp. 1–135.

NOZAWA, A. 1929 The normal and abnormal respiration in the oyster, *Ostrea circumpicta* Pils. Tohoku Univ., Sc. Rep.; Ser. 4, *4:* 315–325.

PARKER, G. H. 1910 The reactions of sponges, with a consideration of the origin of the nervous system. J. Exp Zool., *8:* 1–41.

PATTEN, W. 1894 On the morphology and physiology of the brain and sense organs of *Limulus*. Q. J. Micr. Sc., N. S., *35:* 1–96.

——— 1912 The Evolution of the Vertebrates and their Kin. Blakiston's Sons, Philadelphia. Pp. 1–486.

PATTEN, W., AND W. A. REDENBAUGH 1899 Studies on *Limulus*. II. The nervous system of *Limulus polyphemus* with observations upon the general anatomy. J. Morph., *16:* 91–200.

PETERS, F. 1938 Über die Regulation der Atembewegungen des Flusskrebses *Astacus fluviatilis* Fabricius. Zschr. vergl. Physiol., *25:* 591–611.

PIÉRON, H. 1908 De l'influence de l'oxygène dissous sur le comportement des invertébrés marins. C. rend. Soc. Biol., *64:* 886–888, 955–957, 1020–1022, 1061–1063, 1161–1163.

POLYAK, S. L. 1941 The Retina. University of Chicago Press, Chicago. Pp. 1–607.

PROSSER, C. L. (EDITOR) 1950 Comparative Animal Physiology. Saunders, Philadelphia. Pp. 1–888.

REDFIELD, A. C. 1934 The haemocyanins. Biol. Rev., Cambridge Philos. Soc., *9:* 175–212.

REDFIELD, A. C., T. COOLIDGE AND A. L. HURD 1926 The transport of oxygen and carbon dioxide by some bloods containing hemocyanin. J. Biol. Chem., *69:* 475–509

ROTH, L. M., AND E. R. WILLIS 1951 Hygroreceptors in Coleoptera. J. Exp. Zool., *117:* 451–487.

SCHULMAN, M. P., AND G. WALD 1951 The valence of copper in hemocyanin. Biol. Bull., *101:* 239–240.

SEGAAR, J. 1934 Die Atmungsbewegungen von *Astacus fluviatilis*. Zschr. vergl. Physiol., *21:* 492–515.

STØRMER, L. 1951 Proposed use of the plenary powers to validate the entry on the ''Official List of Generic Names in Zoology'' of the name ''*Limulus*'' Müller, 1785 (class Merostomata): proposed correction of an error in ''Opinion'' 104. Bull. Zool. Nomenclat., Lond., *2:* 319–322.

STUNKARD, H. W. 1951 Observations on the morphology and life history of *Microphallus limuli* n. sp. (Trematoda: Microphallidae). Biol. Bull., *101:* 307–318.

WALSHE, B. M. 1951 Autoecology of *Chironomus plumosus*. Année Biol., Ser. 3, *27:* 555–559.

WATERMAN, T. H., AND D. F. TRAVIS 1951 Respiratory reflexes and the flabellum of *Limulus*. Anat. Rec., *111:* 523 (abstract).

WELLS, G. P. 1951 On the behaviour of *Sabella*. Proc. R. Soc., Lond., *B 138:* 278–299.

WOLVEKAMP, H. P. 1949 Oxygen and carbon dioxide transport by blood containing haemocyanin. Pp. 301–311 in Hemoglobin. F. J. W. Roughton and J. C. Kendrew (Editors). 1949, Butterworths, London. Pp. 1–317.

PLATE 1

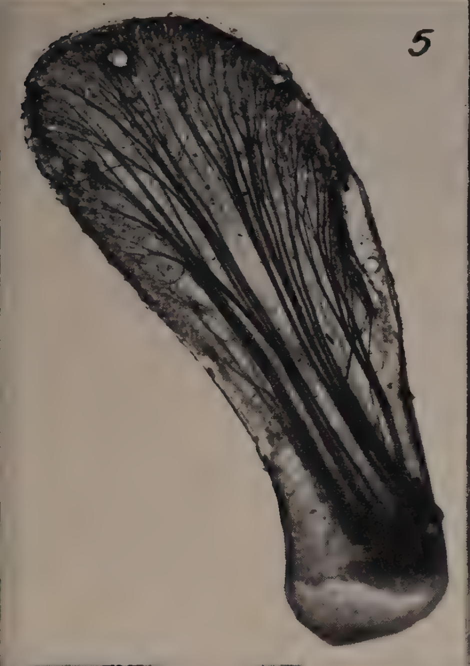
EXPLANATION OF FIGURES

5 Gross innervation of the *Limulus* flabellum, shown in a fixed methylene blue preparation from an immature specimen. The structure is seen from the posteromedial aspect with the distal spatulate blade at the top. In preparation, the integument of the more rounded posterior flabellar surface was removed to expose the distribution of the large nerve supplying the sensory pores on the flat anterolateral surface of the blade. Staining and fixing procedure similar to one described by Polyak ('41, pp. 46 ff.). × 10.

6 Detail showing distribution of flabellar nerve to the special organ on the anterior surface of the blade. The elongate masses of neuron cell bodies and related structures can be seen at the right, supplied by the distal ramifications of the nerve. In the lower right near the margin is an encysted metacercaria of *Microphallus limuli,* a digenetic trematode heavily infesting *Limulus* from certain areas (Stunkard, '51). This flabellum was stained with methylene blue, fixed and dissected out of the integument as a whole mount. × 85.

7 Flabellar detail demonstrating cuticular structures related to the sensory pores of the anterolateral surface of the organ. This surface is seen edge-on at the upper right and includes an area on which three of the pores open. The thin outermost dark layer of the integument is the epicuticle. The thicker light layer beneath this is the procuticle. Under the latter are the dark staining cells of the epidermis. The sensory pores are depressions, here black, in the exocuticle from the bottom of which run cuticular tubules through the procuticle and epidermis to the cell masses beneath (compare fig. 8). The cuticular tubule is surrounded by a sleeve of cells as it traverses the procuticle through a wide pore, and the epidermis. Cross section through the flabellar blade, oriented as in figure 8 and stained with the Masson-Foot-Goldner trichrome technique (Jones, '50, p. 249). Ten micra. × 2000.

8 Cross section of the blade of the flabellum seen with the sensory (anterolateral) surface to the right and dorsoventral orientation vertical. The sensory pits and cuticular canals may be seen in the integument. Immediately beneath is the dark epidermis. Within this are the masses of cells into which the cuticular tubules penetrate (fig. 7) and from which the branches of the flabellar nerve arise (fig. 6). The darker oval areas deep within the flabellum are nerve branches (figs. 5, 6) seen in cross-section. Note that the other (posteromedial) surface of the flabellum is relatively devoid of sense organs in this region. The crumpling of the cuticle of both surfaces is an artifact arising from fixation of a freshly molted organ and subsequent treatment with diaphanol to mollify the material for cutting. Ten micra section, stained with Masson-Foot-Goldner trichrome technique. × 85.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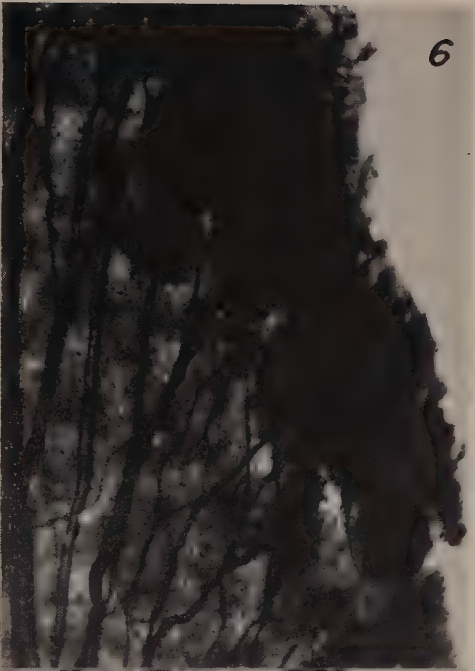
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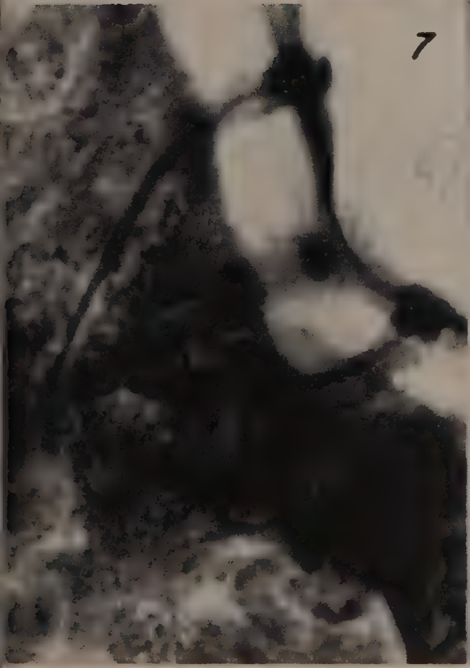
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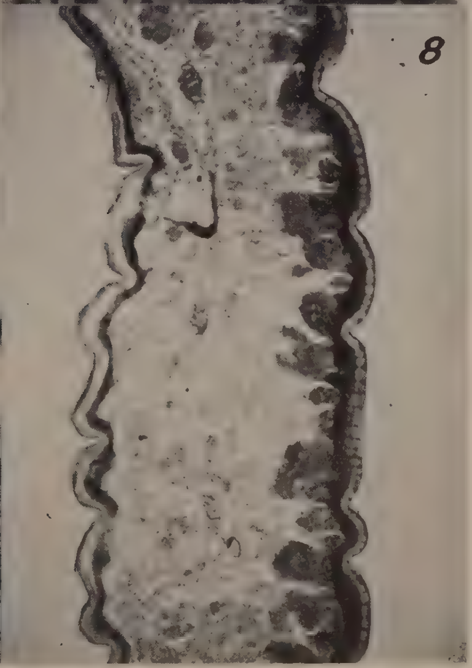
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ATMOSPHERIC RESPIRATION AND THE COMPLEX CYCLES IN MAMMALIAN BREATHING MECHANISMS[1]

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A recent report (McCutcheon, '51) calls attention to elements in mammalian breathing which have not been generally recognized; namely, polyphasic ventilation cycles in the horse and rhythmically recurring complementary cycles in the rat. The complementary cycle was characterized by predictable recurrence in regular sequence among ventilation cycles in resting animals. It consisted of a very deep, rapid inspiratory movement, and an initially rapid expiratory movement which merged into a very slow expiratory movement. During the expiratory movement the expired gas volume was found to be less than the inspired volume by an amount which could not readily be accounted for in terms of respiratory exchange of O_2 and CO_2 or by changes in the mid-point of breathing. Evidence from pressure and volume analyses of these cycles indicated their importance in the intra-pulmonary regulation of diffusion factors at respiratory epithelium and suggested a physiologically modified intra-alveolar pressure in mammals.

Data are now available from a series of mammals on the frequency and duration of complementary cycles to provide additional evidence that they are integral components of the mammalian breathing mechanism. These are reported, and consideration is given to some of the elements in atmospheric respiration which make such cycles appear significant in the physiology of the mammalian pulmonary unit.

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[1] Presented in part at a meeting of the Federation of American Societies for Experimental Biology; Cleveland, Ohio; April 29, 1951.

METHODS AND RESULTS

Data were obtained from tambour recordings of pneumograph, spirometer, or closed chamber as previously described. Results from single individuals of several species of animals (except man) are summarized in table 1, together with related breathing factors taken from the designated reference sources. The data for man are generalized from the references cited.

The difficulty of obtaining standard normal mean values for respiratory and circulatory factors in animals is being recognized (Moses, '46; Owens, '50). The animals here reported, all mature adults, required conditioning to procedures of recording. That the reported animal is a typical normal resting specimen was judged by observation of undisturbed animals in their usual environment and by supplementary use of light urethane anesthesia. Direct visual inspection of breathing and rate counts were used as major indicators of basal conditions. In view of the time and care required to obtain the record of a standard animal, replications of animals or measurements are not here considered. Rather, the most representative mean values for specific normal animals under the most apparently uniform standard resting conditions are given. At least 10 cycles are represented in each mean value, and all values are considered to be within 20% of any standard normal value and normal range of variation to be derived from suitably controlled replication and more extensive statistical considerations.

Certain relationships are of interest in these comparisons, as follows:

1. Ventilation cycle characteristics and complementary cycle frequencies vary with body size in the same general manner.

Breathing factors in terrestrial mammals [1]

ANIMAL		ALVEOLUS [5]	VENTILATION CYCLES			COMPLEMENTARY CYCLES		
Name — Common, Scientific	Weight — kilograms	Depth — microns	Frequency — cycles/min.	Volume — ml	Duration — seconds	Frequency — cycles/hr.	Volume — ml	Duration — seconds
Mouse *(Mus musculus)*	0.0152	39.78	125	0.1 [4]	0.5	45	. .	4
Rat [6] *(Rattus norvegicus)*	0.273	64.98	60	1.4	1.0	26	4.7	5
Guinea pig [6] *(Cavia cobaya)*	0.495	89.07	84	1.9 [4]	0.7	17	. .	7
Rabbit *(Lepus cuniculus)*	3.0	100.57	66	18.3 [4]	0.9	10	. .	6
Cat *(Felis domesticus)*	2.6	148.87	26	12.4 [9]	2.3	6	. .	9
Dog (Pointer) *(Canis familiaris)*	19.2	81.75	14	110.0 [8]	4.3	5	. .	7
Man (Caucasian) *(Homo sapiens)*	70.0	180.90	16 [7]	500.0 [7]	3.5 [7]	3 [2]	. .	8
Horse (Standard bred) [6] *(Equus caballus)*	550.0	. . . . .	12	6000.0 [3]	5.0	0	0.0	0

[1] Standard normal resting mean values at room temperature (24–28°C.). Superscripts are designators of reference.

[2] Erikson, H. (unpublished records, Caucasians and Eskimos on Scholander respirometer, 1949).

[3] Gmelin, L. (from ''Vergleichende Physiologie der Haussäugetiere''; Ellenberger and Scheunert, 1925; Berlin).

[4] Guyton, A. C. (calculated from data in Am. J. Physiol., 1947, *150:* 70–77).

[5] Hartroft, W. S., and C. C. Macklin (unpublished data, reviewed in Anat. Rec., 1947, *97:* 417).

[6] McCutcheon, F. H. (J. Cell. and Comp. Physiol., 1951, *37:* 447–476).

[7] Nims, L. F. (from ''Physiology''; J. F. Fulton, ed.; chap. 39, Respiration; 1949; Philadelphia).

[8] Romijn, C. (calculated from data in ''Collected papers''; Lab. Vet. Physiol., 1947; Utrecht).

[9] Wang, S. C., and L. F. Nims (calculated from data in J. Pharm. and Exp. Therap., 1948, *92:* 187–195).

2. Alveolar dimensions increase with body size in the rodents from mouse through rabbit, but the cat's dimensions approach those of man whereas those of the dog approach those of the guinea pig.

3. Duration of the complementary cycles is correlated with body size in the same manner as alveolar dimensions.

DISCUSSION

Bucher ('49) reports that in a series of 6 species of etherized mammals ranging from mice to man the ventilation varied with surface area but tidal volume and residual volume varied with body weight. A similar trend is apparent for tidal volumes in table 1. The unusual position of the cat in this series, with low ventilation cycle frequency in relation to weight and highest complementary cycle duration, might be correlated with its relatively large alveolar dimensions. The lack of complementary cycles in the horse was previously explained in relationship to the long polyphasic ventilation cycle (5 seconds) which should allow time for intrapulmonary regulation by smooth muscle. Such regulation was previously inferred as the primary significance of complementary cycles in the rat. The range of variation in cycle duration for the other animals (overall mean duration 6.5 seconds) is consistent with this view. Their relatively infrequent occurrence in larger animals probably explains why they have been ignored in studies of breathing. However, examination of breathing records published in the numerous papers on mammalian breathing will show that such cycles are in fact represented in any type of normal and in most experimentally modified breathing if recordings are of sufficient duration. As examples, the paper of Peyser, Sass-Kortsak and Verzar ('50) on volume regulation in breathing shows records for the rabbit and cat which include such cycles; and the paper of Wang and Nims ('48), reporting the effect of anesthesia and decerebration on the CO_2 stimulating action on respiration, shows such a cycle in the cat. Though complementary cycles have been seen by numerous observers of mammalian breathing characteristics, their occurrence has not been remarked, nor their normal relationship in breathing patterns nor their significance in respiratory exchange been suggested.

There are two important papers by Larrabee and Knowlton ('46) which can be related to the significance of complementary cycles in respiratory regulation. These authors report the results of studies of spike potentials in the vagus nerve during experimental alteration of stimuli to pulmonary receptors — those normally designated *volume receptors.* They identify two types of receptors which they call *slowly adapting receptors* and *rapidly adapting receptors.* Regarding the rapidly adapting receptors, they make the following statement:

"The rapidly adapting receptors come into action in appreciable number only when the depth of inspiration is increased. Even with a moderately large increase in depth, however, the discharge consists of not more than a few impulses occurring at the peak of some of the inspirations. Much greater activity is obtained in the very deep inspirations that occur under certain special conditions. For example, it is characteristic of the respiratory movements caused by high CO_2 or low O_2 in the anesthetized cat or dog that an occasional very deep inspiration appears (fig. 14). These inspirations are accompanied by an intense discharge from the rapidly adapting endings (fig. 17)."

After discussing the mechanisms by which these receptors are considered responsible for the deep inspirations, noting that the occasional deeper inspirations do not appear after the vagus nerves have been cut (fig. 14 b); they continue:

"The very deep inspirations recur too infrequently during hyperpnea to contribute much to the minute volume, although even a small increase may be of importance to an individual in extreme respiratory distress. Of greater interest is the fact that impulses discharged from the rapidly adapting receptors can also reinforce deep inspirations under other circumstances. Consequently a very deep inspiration can be evoked by a stimulus whose intensity would cause an inspiration of only moderate depth were it not for this vagal reflex. Isolated deep inspirations occur involuntarily in sneezing, coughing, yawning, and sighing, as well as voluntarily in speaking and singing. A possible mechanism for reinforcing the initial deep inspiration in each of these acts is provided by the autogenous inspiratory reflex.''

The important contributions of Knowlton and Larrabee to the regulatory mechanism of breathing provides the neurological basis for regulation of complementary cycles. Though they appear to attach more significance to secondary features of these deep inspirations in man, the facts reported here show that such deep inspirations normally occur in specific relationship to breathing *per se* in mammals. This points to the likelihood that such acts as sneezing and speaking are ancillary functions of these cycles, and that an intrinsic involvement of the autogenous respiratory reflex in actual respiratory exchange marks the fundamental, primitive significance of such cycles in the evolution of mammalian breathing. They may indeed have to be viewed as the functional reflection of some vestigial neurone complex in larger animals; that is, vestigial functions.

Virtually no information is available about the specific circumstances of diffusion and effusion in the pulmonary unit of the mammalian lung. Data from physiological measurements on man and dog are abundantly available to which such inferential terms as ''alveolar air,'' ''dead space,'' and ''alveolar pressure'' are commonly applied. From this indirect information and from equally valid inferences drawn from comparative physiology of atmospheric respiration among organisms in general, deep inspiratory cycles can be shown to have important influences on regulation of gaseous exchange at the level of the bronchioles, air sacs and alveoli.

It has been established from extensive studies of diffusion of CO_2, O_2 and H_2O, under the conditions prevailing at the stomata of plants (Stiles, '50), that diffusion through pores of 300 μ diameter down to less than 0.5 μ progresses according to the formula $Kp\pi r^2/l + \frac{1}{2}\pi r$, where K is the coefficient of diffusion of gas, p the density of the gas, r the radius of the pore, and l the depth. When pores in a septum are no closer together than 10 times pore diameter, diffusion of CO_2 through a number of pores of 300 μ diameter or less will be at a rate as much as 50 times greater than through an equal area of exposed evaporating surface. The quantity of gas diffusing is proportional to the perimeters rather than the areas of pores. Just this type of relationship prevails between the alveoli and air sacs and between the airs sacs and respiratory bronchioles of the mammalian lung, for the diameters of bronchioles and alveolar mouths are in the order of magnitude at which the ''perimeter law'' comes into operation.

The typical ventilation cycles of mammals can mechanically aerate only the peripheral parts of the lungs. A ''nitrogen space'' can be considered to remain, through which transfer of O_2, CO_2, and H_2O to and from respiratory epithelium must occur. The magnitude of this space is variable. It must be traversed largely by diffusion, with convection, peristaltic activity and ciliary action playing a possible secondary role. But the complementary cycles, involving a mechanically moved volume of air as much as 5 times larger than ordinary ventilation cycles, must markedly alter the characteristics of this space. They will mechanically aerate close to or within most of the air sacs, and thus flush the system down to the alveolar mouths, if not within alveoli. Complementary cycles will thus greatly alter the diffusion characteristics within pulmonary units and they must be important factors in the regulation of respiration.

A point of interest concerning the exchange process in the ''nitrogen space'' is whether an appreciable counter diffusion current through pores can occur against an effusion current. This has been tested in our laboratory by placing N_2

in the bell of a 15 ml Buchner bacteriological filter funnel and flowing a standard O_2–CO_2 (88.8%–10.5%) mixture across the other side. The disc was 1 mm thick with pores 1.2 μ in diameter. The bell was inverted in a cylinder of acidulated water, or in glycerine, to a depth of either 0.1 mm or 270 mm. At 0.1 mm depth no volume change occurred in the funnel due to effusion, but at the end of two minutes the O_2 concentration in the nitrogen was 61% and the CO_2 was 6%. At 270 mm depth the volume in the funnel reduced up to one-half by effusion during two minutes, but the O_2 concentration was 60% and the CO_2 concentration 6% in the remaining gas. Thus diffusion in large quantities occurred counter to effusion currents of considerable magnitude through the numerous pores of the disc. This is the type of exchange process to be considered in the physiology of the pulmonary unit, and much more critical analysis of such factors is needed.

Means to regulate diffusion at respiratory surfaces appears to be a general feature of atmospheric respiration. The most elementary control of this factor is evident in the stomatal guard cells of plants and in the spiracles of those molluscs and arthropods which similarly depend upon diffusion respiration (Krogh, '41). Control of water vapor loss is a major element in these regulations. In animals with active ventilation similar regulatory actions persist, as seen in spiracles of insects and in nares and glottis of amphibians and reptiles. In amphibians (Krogh, '41) and reptiles (McCutcheon, '43; Boelaert, '50) the glottis is normally closed, breathing is periodic, and long periods of apnea permit O_2, CO_2 and H_2O exchange across respiratory epithelium with minimum water vapor loss from the lung to the atmosphere. In adult mammals, however, the glottis is normally open, breathing (ventilation cycles) is essentially uninterrupted and water vapor loss is unchecked at the external entrances to the respiratory passages.

In turtles there is a rythmic pattern of throat movements reminiscent of essential breathing in amphibians and of eupneic breathing patterns in birds and mammals, but for which there is good evidence (McCutcheon, '43; Root, '49) that it is not directly related to ventilation but rather to olfaction. Indeed this throat action was long and erroneously considered to be the essential pulmonary ventilation movement of turtles. It is likely that much of the rythmical mouth and throat movement in reptiles, amphibians and elasmobranchs generally is related to olfaction rather than ventilation, but direct experimental verification is lacking. Olfaction is a primitive sense of great importance in these classes, as indicated by the relatively large development of the olfactory lobes of the brain. The nervous regulation of these movements may well be the evolutionary basis for the control of eupneic and panting types of breathing in mammals, in contrast to their more primitive, periodic sighing type (complementary cycles). Sighing, as a discrete breathing type in mammals, has also been recently emphasized by Hoff and Breckenridge ('52). This type may have its evolutionary neurological origin in the periodic deep ventilation movements of the lower vertebrates.

Regulation of diffusion can only occur at the distal passages of the mammalian lung. Such regulation, based on the differential diffusion and effusion of respiratory gases, is inherent in the sphincter action of pulmonary myoelastic tissue. In addition, all air-breathing vertebrates, unlike water-breathing animals, encounter problems of maintaining an adequate gas-water interphase at the respiratory membranes while living in what is essentially a sea of nitrogen. The compressability and rapid water vapor diffusion characteristics of this gaseous environment create problems which are virtually non-existent in the physiology of aquatic animals. But certainly the control of water, carbon dioxide, and oxygen diffusion is as urgent for birds and mammals as for other organisms with atmospheric respiration.

Previous study of the complementary cycles in rats produced measurements which were used to calculate an approximate intra-alveolar pressure of 20 mm Hg above atmospheric. Assumptions from mammalian data (other than rat) were used to separate the proportion of volume reduction believed due to compression from that due to RQ factors. Specific data have since become available for the rat to verify these assumptions, as follows:

a. Cardiac output (Blood et al., '50)	46.5 ml/min.
b. A–V O_2 difference (Blood, '51, personal communication)	7.1 ml O_2/100 ml
c. Comp. cycle duration	5.0 sec.
d. Comp. cycle blood flow (46.5/60/5)	3.9 ml
e. Comp. cycle O_2 uptake/cycle (3.9 × 0.071)	0.2769 ml
f. Comp. cycle CO_2 output/cycle (0.2769 × 0.8)	0.2215 ml
g. Comp. cycle volume reduction due to normal RQ factors (e-f)	0.0554 ml

The value of 0.0554 ml excess of oxygen uptake over carbon dioxide output therefore agrees with results (breathing coefficient) obtained for rats which were breathing in a closed chamber (0.73–1.08 ml/min.; or 0.06–0.09 ml/cycle). However, such results are based on exchanges during typical ventilation cycles; they do not incorporate variations in oxygen uptake and CO_2 output such as those shown to occur in deep breaths and in breath-holding in man.

Armitage ('49) gives evidence that a single deep breath in man can double the normal oxygen consumption over that of a typical ventilation cycle, presumably as a result of intrapulmonary circulatory increase; Stevens ('46) showed from serial samples of arterial blood gas and pH in man, that during breath-holding, oxygen moves readily from lung to blood while carbon dioxide movement in the opposite direction is small, which is related to the high CO_2 capacity of the body and the nature of the HbO_2 dissociation curve. The use of normal RQ factors to arrive by calculation at an intra-alveolar pressure value is, therefore, subject to considerable error, as previously indicated. If the inference of pressures other than atmospheric were to rely on this procedure alone, its validity would be correspondingly doubtful. Other evidence from the nature of the pneumograms, pressure variation in the actual or potential gas spaces in the body, effects of altered regulatory elements, and the comparative physiology of the breathing mechanism must also be recognized.

Explanation, in terms of specific intra-pulmonary events, of the results of deep breaths and of breath-holding, diving, and similar variations from typical eupneic ventilation cycles is difficult; for assumptions must be made about the details of exchange within the pulmonary unit. Though breath-holding in man and diving in many animals have been extensively studied, the resulting data are not directly applicable to complementary cycles, since the evidence shows that the trachea is normally open to the atmosphere during these cycles.

It does not seem warranted, therefore, to attempt at this time a refinement of the analysis of intra-alveolar pressure based on such pertinent but inadequate information. The primary purpose of this report is to emphasize the normal occurrence, distribution, characteristics and significance of the sigh-like complementary breathing cycles among mammals.

SUMMARY AND CONCLUSIONS

Data are summarized for 8 mammals (mouse, rat, guinea pig, rabbit, cat, dog, man, horse) to correlate complementary cycles with other elements of the breathing mechanism. The results show that such cycles regularly occur in all these mammals except the horse.

The cycle frequency varies with characteristic body size among the species in the same manner as typical ventilation cycles, the cycle frequency during an hour for the respective species being 45, 26, 17, 10, 6, 5, 3, 0. However, complementary cycle duration is quite uniform, the duration in seconds for the respective species being 4, 5, 7, 6, 9, 7, 8, 0 (mean — 6.5 seconds). Thus duration of cycles varies more nearly with alveolar dimensions than with body size.

The lack of complementary cycles in the horse may be compensated by the slow (5 second) polyphasic ventilation cycle. This is consistent with evidence that intra-pulmonary regulation of diffusion and pressure is exerted by intrinsic myoelastic tissue. It is suggested that diffusion regulation in respiration is a principle of the comparative physiology of atmospheric respiration for organisms in general, and that periodic breathing is the primitive pattern of atmospheric ventilation.

LITERATURE CITED

ARMITAGE, G. H., AND W. M. ARNOTT 1949 Dead space and alveolar gas tensions. J. Physiol., *109:* 64–69.

BLOOD, F. R., D. L. SMITH AND F. E. D'AMOUR 1950 Cardiac output in rat at normal and at high altitudes in relationship to gas embolism. Am. J. Physiol., *163:* 268–271.

BOELAERT, R. 1950 Sur la physiologie de la respiration des Lacertiens. Arch. Internat. Physiol., *51:* 379–437.

———— 1950 Sur la physiologie de la respiration de *l'Alligator mississippiensis.* Arch. Internat. Physiol., *52:* 57–72.

BRECKENRIDGE, CARLYLE G., AND HEBBEL E. HOFF 1952 Influence of morphine on respiratory patterns. J. Neurophysiol., *15:* 57–74.

BUCHER, K. 1949 Pulmonary ventilation. Helv. Physiol. et Pharmacol., *7:* 470–475.

HOFF, HEBBEL, AND CARLYLE G. BRECKENRIDGE 1952 Levels of integration of respiratory patterns. J. Neurophysiol., *15:* 46–56.

KNOWLTON, G. C., AND M. G. LARRABEE 1946 A unitary analysis of pulmonary volume receptors. Am. J. Physiol., *147:* 100–114.

KROGH, A. 1941 The comparative physiology of respiratory mechanisms. Philadelphia.

LARRABEE, M. G., AND G. C. KNOWLTON 1946 Excitation and inhibition of phrenic motoneurones by inflation of the lungs. Am. J. Physiol., *147:* 90–99.

McCUTCHEON, F. H. 1943 The respiratory mechanism in turtles. Physiol. Zool., *16:* 255–269.

———— 1951 The mammalian breathing mechanism. J. Cell. and Comp. Physiol., *37:* 447–476.

MOSES, L. E. 1946 Heart rate of the albino rat. Proc. Soc. Exp. Biol. and Med., *63:* 58–62.

OWENS, O., AND W. H. GANTT 1950 Does presence of a person act on cardiac rate of the dog as unconditioned stimulus? Am. J. Physiol., *163:* 740.

PEYSER, E., A. SASS-KORTSAK AND F. VERZAR 1950 Influence of O_2 content of inspired air on total lung volume. Am. J. Physiol., *163:* 111–117.

ROOT, RAYMOND W. 1949 Aquatic respiration in the musk turtle. Physiol. Zool., *22:* 172–178.

STEVENS, C. D., E. B. FERRIS, J. P. WEBB, G. L. ENGEL AND M. LOGAN 1946 Voluntary breathholding. I. Pulmonary gas exchange during breathholding. J. Clin. Invest., *25:* 723–728.

STILES, W. 1950 Principles of plant physiology. Methuen, London.

WANG, S. C., AND L. F. NIMS 1948 The effect of various anesthetics and decerebration on the CO_2 stimulating action on respiration in cats. J. Pharm. and Exp. Therap., *92:* 187–195.

EFFECTS OF SHEATH REMOVAL ON BULLFROG NERVE

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ONE FIGURE

INTRODUCTION

Recent studies (see Crescitelli, '51; and Feng et al., '50; for references) have noted a marked increase in the rate of penetration or of action of diffusible substances in nerve when the external sheath (epineurium) is removed. This is attributed to the elimination of the major resistance to diffusion which is assumed to reside in the sheath. Lorente de Nó ('50) rejects this conclusion on the basis of the rapidity with which effects appear in the intact trunk when diffusion gradients are small.

In the course of research on desheathed bullfrog nerves we have found they undergo changes which make them undesirable at least for certain problems. This was briefly reported at a recent symposium (Shanes, '51a). The findings may modify the interpretation of results based on such preparations. Consequently, although by no means exhaustive, they appear of sufficient importance and clarity to justify this brief communication.

METHODS

Sciatic nerves from Rana catesbiana were used exclusively. The sheath was removed from the tibialis and peroneus branches and the proximal unbranched segment from which they arise. This was accomplished in a single operation by girdling the nerve in a still more proximal area, cutting intervening branches very short, and drawing the sheath distally with care, as described by Feng and Liu ('49).

The ends of the nerves were ligated with thread, which served for handling purposes. In weighing experiments, nerve and thread were gently blotted on filter paper and quickly weighed on a 250 mg Roller-Smith torsion balance with a vernier of 0.1 mg/division. Reproducibility of weighings was within 1.5%.

The first weight determination, corresponding to zero time in figure 1, was made immediately upon sheath removal; if the nerve is immersed in Ringer's first — as apparently was the case in the experiments of Feng and Liu ('49, p. 12) and in some of our earlier experiments — water uptake is so rapid initially that a large part of the weight gain may easily be missed. The considerably smaller figure for swelling (10%) reported by these investigators may be accounted for in this way, although the possibility of a species difference is by no means ruled out.

Analyses for sodium and potassium were carried out as previously described (Shanes, '52). Thus, the nerves were weighed before and after drying to constant weight, incinerated for 16 hours at 500°C., and an aqueous extract was prepared from the ash. The Beckman 103000 flame spectrophotometer served for analysis of both sodium and potassium in the same samples from individual nerves. The average deviation of individual readings usually was ½%. The use of standards with both sodium and potassium concentrations equivalent to those in the unknowns, and of the paired nerve technique, gives highly reproducible results. The statistical problem was therefore negligible.

Action potentials were observed with oxygenated medium circulating past the nerves as previously described (Shanes, '51b). Electronic equipment was conventional.

RESULTS

Weight changes. Removal of the sheath causes a dramatic increase in weight. The typical time course is illustrated in figure 1. About half the increment, which is practically maximal in 3 to 4 hours, is completed within the first 20 minutes. The weight increase in 3 to 4 hours is about 40% (table 1).

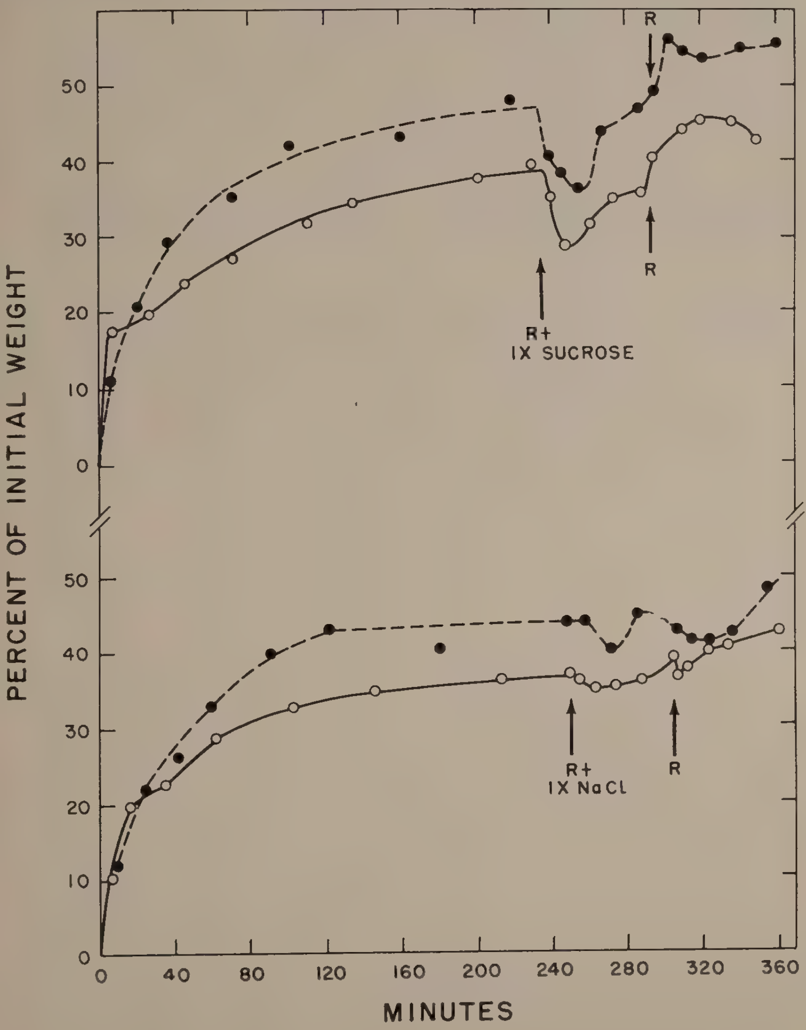

Fig. 1 The percentage increase in weight, relative to the initial, of desheathed nerve measured from the time of sheath removal. The osmotic response to Ringer's (R), with the tonicity doubled with either excess sucrose or sodium chloride, also is shown. Curves with the same symbols are from paired nerves.

The osmotic response to hypertonic Ringer's is poor in the late stages of swelling. Thus, in Ringer's containing an additional 110 μM/ml of sodium chloride, shrinkage is negligible. When an osmotically equivalent excess of sucrose is used instead, the weight loss, while larger, is less than in intact nerve (Shanes, '48a, b) and transitory. These characteristics are apparent in figure 1.

TABLE 1

A comparison of the sodium and potassium contents of intact and desheathed nerve, and the increase in weight, relative to the initial, 4 hours after sheath removal

	WEIGHT INCREMENT	SODIUM CONTENT		POTASSIUM CONTENT	
		Intact	Desheathed	Intact	Desheathed
	%	μM/gm final weight		μM/gm final weight	
	42	82.5	89.8	34.4	29.4
	35	77.6	91.3	40.7	33.8
	44	79.8	94.3	32.7	20.5
	48	71.2	87.6	33.4	22.0
	41	74.2	91.6	30.4	20.3
	37	70.6	80.8	34.8	25.5
Mean:	41	75.9	89.2	34.4	25.3

Electrolyte changes. The poor osmotic response of desheathed nerve may indicate damage as a result of the initial swelling or greater susceptibility to injury under conditions of hypertonicity. The potassium content of nerve is sensitive to damage, consequently its determination might indicate whether the first possibility is correct.

Table 1 shows that the potassium, referred to the final weight, is consistently lower in desheathed nerves. Since water has been taken up to the extent of 40%, the potassium figure must be multiplied by 1.4 to determine whether a change has actually occurred. The result is 35 μM/gm initial weight. Therefore, the fibers have lost no potassium despite the swelling of the trunk and by this criterion are undamaged.

The sodium level is increased by desheathing (table 1). This is explainable as the result of the uptake of Ringer's equal to the weight gain. Thus, 0.4 ml Ringer's contains 43 μM of sodium; its entry into 1 gm of nerve will therefore give (76 + 43)/1.4 or 85 μM/gm final weight, which is close to the figure actually obtained.

Functional changes. The ability of desheathed nerve to withstand prolonged repetitive activity, demonstrated in table 2, is at least as good as that of intact nerve. Moreover, the aerobic reabsorption of potassium following anoxia is more readily demonstrated in desheathed preparations (Shanes, '51a). The functional integrity of the fibers shown by these observations is consistent with the indications of the potassium data.

TABLE 2

Comparison of spike heights at the beginning and at the end of bouts of activity in intact and desheathed nerve

FREQUENCY OF STIMULATION	DURATION OF STIMULATION	RELATIVE SPIKE HEIGHT			
		Desheathed nerve		Intact nerve	
Shocks/sec.	*Min.*	*Initial*	*Final*	*Initial*	*Final*
100	20	17	16.5		
		7.5	6.0		
		13.5	12	14.5	9
100	60	24	16	28	19
50	10	14.5	14	15	13
25	20	13	10		
		9.5	9		
25	60	18	17	28	25

DISCUSSION

The available data suggest that in desheathed nerve the fibers remain relatively intact while the extracellular space takes up a substantial amount of medium. This is consistent with Lorente de Nó's histological findings, which indicate unchanged axoplasmic diameters but an increase in the interfibrillar space and in the thickness of the sheaths ('52, and personal communication). Results obtained by Mullins and Grenell ('52) likewise suggest an increase in the extracellular space.

A possible explanation of the swelling is that removal of the sheath eliminates a physical restraint which normally counteracts the osmotic imbalance characteristic of a Donnan equilibrium in the connective tissue. If such an equilibrium is present it does not seem to involve an amphoteric indiffusible ion like protein, for pretreatment of nerves with a Ringer's brought to a pH of 5 with acid phosphate failed to reduce water uptake.

In any case, enlargement of the extracellular space must be taken into account in interpretations of the effects of sheath removal. Thus, improved penetration may be due to increased spacing between fibers as well as to the absence of the sheath *per se*. The importance of spacing has come to our attention in analytical studies confirming and extending those of Fenn et al. ('34) on the intact trunk (Shanes and Berman, in preparation). The relative impermeability of the fibers to electrolytes and polar substances generally requires that diffusion occur in the spaces between them. The fibers in the intact trunk appear tightly packed as seen in cross-section (Lorente de Nó, '52), hence the effective area available for penetration (i.e., the interfibrillar distances as compared with the total circumference) is low.

These considerations make apparent an objection to the view that all substances should penetrate nerve at rates comparable to CO_2 (Lorente de Nó, '50). This gas is well known to penetrate cells readily, consequently the fibers as well as the space between them would offer a diffusion path and lead to faster entry than in the case of ions and sugars. Such a difference in diffusion rates has been known for some time (cf. Fenn, '28; and Fenn et al., '34).

If the small interfibrillar spaces are the major cause of the diffusion resistance, Lorente's demonstration of the rapid initiation of effects in intact nerve with small concentration gradients becomes compatible with the more rapid effects obtainable upon removal of the sheath. The fact remains, however, that penetration of the fiber-containing core is a slow process for most substances — about one three-hundredth that in free aqueous solution (Shanes and Berman, in preparation) — consequently inferences drawn with the opposite assumption (see Lorente de Nó, '52, for references) are in need of revision.

SUMMARY

Desheathed bullfrog nerve gains weight up to 40% of the initial in 3 to 4 hours. The potassium content remains unchanged while the sodium level rises to an extent equivalent to the entry solely of Ringer's solution. The ability to fire repetitively is unimpaired. These results suggest that the weight change is an extrafibrillar phenomenon and leaves the fibers themselves relatively intact.

LITERATURE CITED

CRESCITELLI, F. 1951 Nerve sheath as a barrier to the action of certain substances. Am. J. Physiol., *166:* 229.

FENG, T. P., C. H. HSU AND Y. M. LIU 1950 Correlation of potassium movement into and out of the nerve with its depolarization and repolarization. Chin. J. Physiol., *17:* 281.

FENG, T. P., AND Y. M. LIU 1949 The connective tissue sheath of the nerve as effective diffusion barrier. J. Cell. and Comp. Physiol., *34:* 1.

FENN, W. O. 1928 The carbon dioxide dissociation curve of nerve and muscle. Am. J. Physiol., *85:* 207.

FENN, W. O., D. M. COBB, A. H. HEGNAUER AND B. S. MARSH 1934 Electrolytes in nerve. Am. J. Physiol., *110:* 74.

LORENTE DE NÓ, R. 1950 The ineffectiveness of the connective tissue sheath of nerve as a diffusion barrier. J. Cell. and Comp. Physiol., *35:* 195.

——— 1952 Potassium and sodium ions in nerve physiology. Cold Spr. Harb. Symp. Quant. Biol., in press.

MULLINS, L. J., AND R. G. GRENELL 1952 The influence of the axon sheath on phosphate diffusion. J. Cell. and Comp. Physiol., *39:* 1952.

SHANES, A. M. 1948a Medium concentration in relation to the water content and electrical properties of nerve. Fed. Proc., *7:* 111.

——— 1948b Metabolic changes of the resting potential in relation to the action of carbon dioxide. Am. J. Physiol., *153:* 93.

——— 1951a Factors in nerve functioning. Fed. Proc., *10:* 611.

——— 1951b Potassium movement in relation to nerve activity. J. Gen. Physiol., *34:* 795.

——— 1952 Ionic transfer in nerve in relation to bioelectrical phenomena. Ann. N. Y. Acad. Sci., *55:* No. 1.

THE VARIATION IN PHOSPHORUS COMPOUNDS DURING METAMORPHOSIS OF THE BLOW-FLY, CALLIPHORA ERYTHROCEPHALA MEIG

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THREE FIGURES

The fate of phosphorus (P) containing compounds during the metamorphosis of insects is of special interest in view of the importance of these materials in intermediary metabolism. It is probable that the majority of such compounds in insects are identical with those more widely studied in other forms; thus, the adenosine triphosphoric acid (ATP) isolated from *Drosophila* by Albaum and Kletzkin ('48) and from locust muscle by Calaby ('51) is identical with the ATP of mammalian tissue. The ribose nucleic acid (RNA) of blowfly larvae characterized by Khouvine and Gregoire ('44) is identical with that of yeast. The insect phosphagen, arginine phosphate, has been found to be the same as in other invertebrates (Baldwin and Needham, '33). Further, Hassett ('51) has shown that a number of phosphorylated sugars which are intermediates in the glycolytic cycle are rapidly utilized by cockroach tissues, indicating that these P-esters occur normally as components of carbohydrate metabolism.

During adult development, marked changes occur in both the distribution and kind of P compounds present in insects. Heller ('31) showed that the level of inorganic P in the blood of the diapausing hawk-moth *(Deilephila euphorbiae)* pupa was inversely proportional to the U-shaped respiration curve.

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This work was later extended to include the distribution and amounts of inorganic P, phosphagen P, easily acid labile P (i.e., 7 min. hydrolyzable P assumed to be ATP), and total trichloroacetic acid (TCA) soluble P in the various tissues of the larval, pupal and adult stages (Heller, '36; Heller, Swiechowska and Karpiak, '49). From these studies a correlation was drawn between the concentration of easily hydrolyzable P and the metabolic rate at the various stages of development. The changes in 4 types of P compounds were investigated during the metamorphosis of *C. erythrocephala* by Khouvine and Gregoire ('40); they found that, expressed on a weight basis the total P remained constant during the whole of adult development, but quantitative changes occurred in the different P fractions. Their results will be discussed in greater detail below.

In the present work, the variation in P compounds during adult development of the blow-fly has been determined by methods more recently developed than those employed by Khouvine and Gregoire ('40), permitting of a somewhat more definite characterization of these materials. Although the data to be presented are as yet incomplete, sufficient experiments have been performed to warrant the drawing of certain conclusions, and to indicate the nature of the problems remaining to be solved.

MATERIALS AND METHODS

The *C. erythrocephala* pupae used in the present experiments were obtained from a culture which had been in-bred in this laboratory for many generations. Two days after the fully grown larvae had left the meat on which they had been raised, the culture was examined at intervals of about two hours, and those showing the first signs of "rounding up" were picked out and termed pupae of zero age. Such pupae were either used immediately, or allowed to develop in a dish of sawdust at 24°C. Some 20–30 pupae of known age, and weighing about 1.5 gm were used for a single experiment, while for a complete series of determinations all pupae at the various stages of development were obtained from a single batch of some hundreds of larvae. The total period for adult development was about 240 hours.

The method of P fraction, based upon the techniques of Schmidt and Thannhauser ('45) and of Schneider ('45, '46), was as follows:

The weighed pupae were homogenized in *c.*5 volumes of ice-cold 10% (w/v) TCA, and the homogenate allowed to stand for 15–20 minutes in the ice chest. Following centrifugation, the supernatant fluid was filtered through a sintered glass funnel containing a layer of Hyflo-Supercell which had previously been washed with dilute TCA and ethanol until free of P. The remaining precipitate was twice treated as above with 2.5% (w/v) TCA and filtered through the original filter cake. Subsequent washing did not extract any more TCA soluble P. The combined water-clear filtrates were neutralized with 10% (w/v) sodium hydroxide (pH 8.2), and the composition of the P esters was investigated either before or after precipitation with barium or ethanol (Umbreit, Burris and Stauffer, '48) by means of the rates of hydrolysis of the various esters according to Lohmann ('28).

The residue remaining from the above treatment was extracted 4 times at room temperature with absolute ethanol-ether (3:1 v/v), both freshly distilled, and the extracts filtered through the same Hyflo-Supercell cake. The filtrates were made up to known volume, and aliquots evaporated to dryness and analyzed for total P. This fraction represented lipid P.

The remaining tissue was completely dried *in vacuo* and analyzed for nucleic acid P by either the Schmidt and Thannhauser ('45) or Schneider ('46) procedure. The values by the latter method, although substantially the same, were generally slightly lower than by the former.

P_o represents the "apparent" inorganic P, and includes any orthophosphate which may have been liberated from particularly labile P compounds by the Allen ('40) procedure employed for the P estimation. "True" inorganic P was measured after its precipitation with $CaCl_2$-Ca $(OH)_2$ as described by Umbreit et al. ('48). $P_{10\ min.}$ and $P_{180\ min.}$ represent the "apparent" inorganic P values after acid hydrolysis for 10 and 180 min. respectively. Total carbohydrate (TC) was determined according to Morris ('48) and the total reducing value (TRV) by the Miller and Van Slyke ('36) procedure.

The data are expressed in mg P per 100 gm fresh weight pupae.

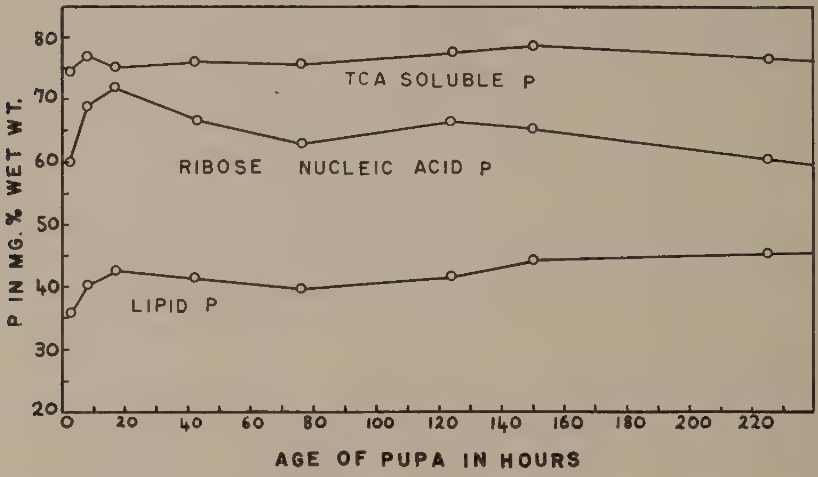

Fig. 1 The variation in TCA soluble P, ribose nucleic acid P and lipid P during metamorphosis of the blow-fly.

RESULTS

The total P of the *Calliphora* pupae — 191 mg % — was quantitatively accounted for by P compounds soluble in TCA and ethanol-ether and by nucleic acid P. The finding of Khouvine and Gregoire ('40) that the puparium (i.e., the pupal case formed from the mature larval skin) contained only traces of P was confirmed, and no evidence was obtained for the presence of any protein bound P. The variation in acid soluble P, lipid P and nucleic acid P with development is shown in figure 1.

There was a gradual increase in the P of all three fractions during the first 18 hours, followed by a more or less slight decrease, after which there was but little change during the rest of metamorphosis. These early changes in the P of all three fractions probably reflect a weight change in the pupae, and may perhaps be correlated with the fact that at about 18 hours there occurs the so-called pupal moult which initiates the true onset of adult development.

RNA-P accounted for 90–95% of the total nucleic acid P in the pupae and no DNA could be detected at any stage in either the dehydrated tissue residue or in pupal extracts employing the Dische ('30) color reagent for desoxyribose. However, of the 5–10% of nucleic acid P unaccounted for, a part is undoubtedly due to DNA-P (see Discussion).

Fractionation of the TCA soluble P

In view of the findings that inorganic pyrophosphate (Heller, Karpiak and Zubikowa, '50) and metaphosphate (Niemierko and Niemierko, '50) (both of which yield inorganic orthophosphate on 10 min. acid hydrolysis), can be formed by certain insects, tests were made to see whether either of these materials was present in *Calliphora.* TCA extracts prepared from *c.*1.5 gm pupae at three different ages were employed for this purpose. No precipitate was obtained upon the addition to the extracts of either lead nitrate at pH 2 (Mann, '44), or of cadmium acetate at pH 3.5 (Cohn and Koltkoff, '42). These results indicate the absence of any significant quantities of inorganic pyrophosphate. The probable absence also of inorganic metaphosphate was demonstrated by the lack of any precipitate in the presence of $Ba(NO_3)_2$ at pH 2 (Juni, Kamen, Reiner and Spiegelman, '48), or of any metachromatic effect on the addition of toluidine blue at pH 4 (cf. Niemierko and Niemierko, '50). It may be concluded, therefore, that any easily hydrolyzable P in the TCA extracts is due to organic P esters.

The changes occurring during metamorphosis in the inorganic P and various P esters as characterized by their rates of hydrolysis in N HCl at 100°C. are shown in figure 2.

The inorganic P was lowest at the commencement of pupation, increased progressively to a maximum value about half way through adult development, and then gradually decreased up to the time of adult emergence. Conversely, the easily hydrolyzable P showed just the opposite change, the amount being high at the beginning and end of metamorphosis, and least about a third of the way along. The non-hydrolyzable P ($P_{\text{total acid sol.}} - P_{180\text{ min.}}$) decreased steadily during adult development, while the value for difficultly hydrolyzable P ($P_{180\text{ min.}} - P_{10\text{ min.}}$) showed irregular fluctuations and was at all times low; the amount was relatively high at the commencement of pupation, decreased to almost zero half way through development, and then increased abruptly to the original value some hours later.

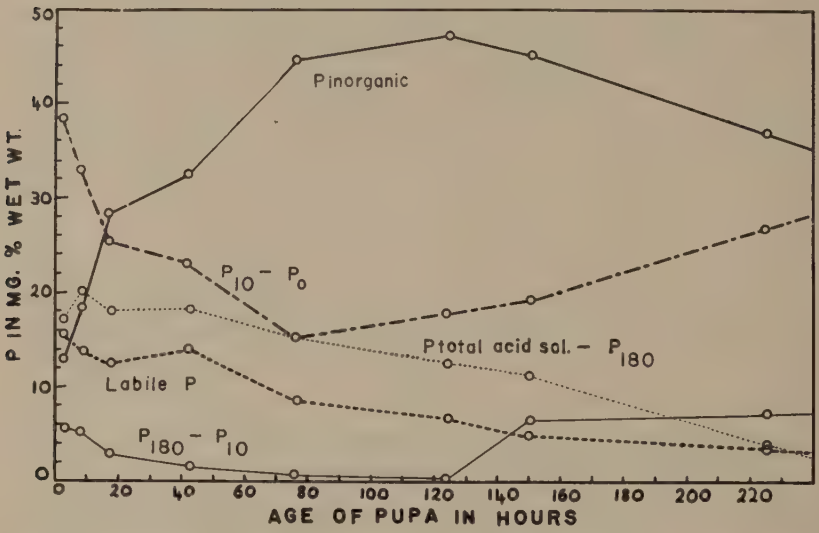

Fig. 2 Variation in the composition of the TCA soluble P of the blow-fly during metamorphosis.

When the orthophosphate was estimated in the usual manner, it was observed that after addition of the reagents employed for P determination, a progressive increase in inorganic P occurred on allowing the solution to stand for 20–25 min. at room temperature. The addition of either molybdate or the reducing agent did not affect the rate of increase in P. This latter finding indicated that the breakdown of creatine P was not involved, since the rate of hydrolysis of the phosphagen is markedly accelerated by the presence of molybdate, and no creatine could be detected in the acidified extracts by Walpole's ('11) diacetyl reaction. The hydroxamic acid test for acetyl P (Lipmann and Tuttle, '45) was also negative. It is presumed, therefore, that some as yet unidentified extremely labile P ester was present, characterized by being completely hydrolyzed by 2.5 N acid at room temperature in 20–25 min., or by .880 ammonia overnight at 0°C. It may be seen from figure 2 (labile P curve) that this material is present in highest concentration at the onset of pupation, and decreases almost to zero by the time of adult emergence.

TABLE 1

Fractionation of TCA and soluble P from Calliphora pupae

For further details see the text

AGE OF PUPA	8 HR.		100 HR.		210 HR.	
	Ba^{++} insol. mg %	Ethanol insol. mg %	Ba^{++} insol. mg %	Ethanol insol. mg %	Ba^{++} insol. mg %	Ethanol insol. mg %
P_o	16.6	0.64	30.4	9.9	20.9	10.0
Labile-P	0.4	9.9	1.2	4.8	...	...
$P_{10} - P_o$	14.2	14.6	5.2	10.0	13.1	12.3
P_{total}	37.6	38.4	37.0	40.8	38.4	36.2

Fractionation of 10 min. hydrolyzable P

Fractionation of the neutral TCA pupal extracts with Ba^{++} and ethanol yielded the data shown in table 1, which summarizes the results of several experiments. Only during the early stages of metamorphosis could the inorganic P be quantitatively precipitated with Ba^{++}; during the later stages considerable amounts also appeared in the ethanol insoluble fraction.

The present procedure was not entirely satisfactory for separation of the labile P esters, although for the most part it was present in that fraction which was Ba^{++} soluble, but insoluble in ethanol. Both the total P and the easily hydrolyzable P were approximately equally distributed in the Ba^{++} and ethanol precipitates. Neither fructose nor any alkali labile P could be detected in either the original TCA extracts or any fraction thereof. The amount of P remaining after Ba^{++} and ethanol precipitation was at all times negligible.

Total carbohydrate (TC) and reducing value (TRV)

The TC and TRV of the *Calliphora* pupae were examined at three different stages of development, with the aim of gaining further insight into the nature of the P esters.

A comparison of the data shown in the first lines of tables 2 and 3 indicates that both TC and TRV (expressed as glucose) were highest at the onset of pupation, and least at the time of adult emergence. Furthermore, the TRV accounted for only about 25% of the TC at the beginning of metamorphosis, and less than 12% at the end. These data suggest that free glucose or other reducing sugars were not present in high concentration, and this was confirmed by yeast fermentation. Following two hours' incubation of neutralized TCA extracts with Baker's yeast it was found that, on the average, only about 9% of the TRV and 2% of the TC could be ascribed to free fermentable sugar, as shown in table 2.

The result of hydrolyzing the TCA extracts of early pupae for 10 min. with N HCl was to increase the TRV from its original value of 435 mg % to 1,260 mg %, and the fermentable reducing value from 40 mg % to 490 mg %. There was no change in TC following acid hydrolysis. Lengthing the period of hydrolysis to 120 min. served to raise the TRV to the TC level of over 1,600 mg %, and to increase the fermentable reducing value still further to 1,309 mg %.

These data would seem to indicate that a non-fermentable polysaccharide, such as glycogen, was being hydrolyzed to yield fermentable reducing sugar; however, it appears probable from the following experiments that glycogen is not present in *Calliphora* pupae.

The addition of 1.5–2 volumes of ethanol to either TCA or boiling water extracts of 24-hour pupae, or to hot KOH pupal digests (as employed in the Pflüger method for the isolation of glycogen), resulted in a flocculent white precipitate and a chocolate-brown supernatant. After washing this precipitate with 70% (v/v) ethanol on the centrifuge it had the following properties: it was only partly soluble in hot water, though freely soluble in cold dilute HCl. The acid solution was not reducing either before or after hydrolysis in 0.6 N HCl for 120 min., neither did it give a positive reaction for carbohydrate with the anthrone reagent (Morris, '48). P was scarcely detectable in the precipitate, while the nitrogen content of the small hot water soluble fraction was 1.8%, and of the larger water insoluble fraction 0.54%. It may be concluded, therefore, that even though the nature of this

TABLE 2

The effect of yeast fermentation on reducing value and total carbohydrate of Calliphora pupa. Age of pupae, 13 hours

	TOTAL REDUCING VALUE (MG % GLUCOSE)	TOTAL CARBOHYDRATE (MG % GLUCOSE)
In TCA extract	436	1,655
do, after yeast fermentation	396	1,620
TCA extract hydrolyzed in N HCl for 10 min. at 100°	1,260	1,715
do, after yeast fermentation	770	...
TCA extract hydrolyzed in N HCl for 120 min. at 100°	1,700	1,615
do, after yeast fermentation	391	...

precipitate remains as yet obscure, it is almost certainly not glycogen.

In table 3 the results are shown of determinations of TC and TRV on the Ba^{++} and ethanol fractions of TCA extracts as employed for the P separation.

The Ba^{++} precipitate contained some 20% of the TRV and 33% of the TC, the corresponding figures for the ethanol precipitate being *c.* 60% and 15% respectively. About 50% of the TC and an amount of reducing material which appeared to vary inversely with the Ba^{++} insoluble TRV were not precipitated by either Ba^{++} or ethanol, although both the TRV and TC could be removed almost completely by the copper-lime treatment of Van Slyke ('17). The increase in TRV following 10 min. hydrolysis of the Ba^{++} insoluble fraction was consistently greater than the corresponding increase in the ethanol fraction, but the results in both cases were very variable. Insufficient experiments have as yet

TABLE 3

The effect of acid hydrolysis and treatment with barium and ethanol of TCA extracts of Calliphora pupae on reducing value and total carbohydrate. Age of pupae in parenthesis

	TOTAL REDUCING VALUE (MG % GLUCOSE)		TOTAL CARBOHYDRATE (MG % GLUCOSE)	
	(100)	(210)	(100)	(210)
In TCA extract	258	114	1,070	980
do, after hydrolysis in N HCl for 10 min. at 100°	580	259	...	...
Barium precipitate	48	...	346	...
do, after hydrolysis in N HCl for 10 min. at 100°	188	...	...	...
Ethanol precipitate	157	103	156	163
do, after hydrolysis in N HCl for 10 min. at 100°	183	228	...	...
Not precipitable by Ba++ or ethanol	53	...	584	...

been performed to determine the cause of this variation, or whether it may be related to different stages of development.

Chromatography of TCA pupal extracts

The results of several attempts to identify the carbohydrates in the TCA pupal extracts by paper chromatography according to the method of Partridge ('48) were for the most part unsuccessful. The neutralized TCA extracts, after concentration, were unsuitable for paper chromatography due to the presence of excess salts. Although these could be removed by the use of ion exchange columns (Partridge, '48), some 50% or more of the TC was retained on the columns, and could not easily be eluted off. The de-salted extracts showed only a very weak spot on the chromatograms, corresponding in position to glucose. After acid hydrolysis for 10 min. in N HCl the glucose spot was somewhat intensified; increasing the period of hydrolysis to 120 min. produced a further slight intensification of the glucose spot, and a second very weak spot due to an as yet unidentified pentose frequently appeared. A known amount of glucose added to the original TCA extracts was satisfactorily recovered on the chromatograms after passage through the ion exchange columns and subsequent acid hydrolysis. Results similar to the above were obtained following chromatography of concentrated, aqueous pupal extracts deproteinized by the chloroform-octyl alcohol method of Sevag, Lackman and Smolens ('38).

To confirm the above evidence that little free sugar was present an experiment was set up as follows:

Pupae 24 hours old were extracted with boiling water, and the turbid solution deproteinized by repeated treatments with chloroform-octyl alcohol. The deproteinized solution — containing 65 mg TC expressed as glucose — was concentrated to one-fifth of the original volume, and ethanol to a final concentration of 60% (v/v) added. The resulting precipitate contained no carbohydrate, and was discarded. The ethanol concentration was then increased to 80% and a further precipitate obtained. After washing with 80% ethanol, this precipitate contained 14 mg TC; the remaining carbohydrate material (50 mg) was present in the supernatant fluid. The ethanol was evaporated off *in vacuo,* and the resulting aqueous solution, together with the above precipitate, were then separately subjected to qualitative tests for sugars employing such reagents as α-naphthol, orcinol or resorcinol in HCl. It was found that the precipitate showed a faintly positive pentose reaction, while the aqueous solution gave a weakly positive test for hexose.

DISCUSSION

The nature of the P compounds in Calliphora pupae

The P compounds in *C. erythrocephala* pupae cannot be precisely characterized from the present work, but the probable identity of some of them may be inferred. On the assumption that the composition of the TCA soluble P is similar to that of mammalian tissues, the following esters are likely to be present.

Easily hydrolyzable P was precipitated by both Ba^{++} and ethanol, but at no stage could any fructose be detected; therefore hexose-di-P, fructose-1-P and fructose-6-P are either absent or are present in undetectable amounts. The Ba^{++} precipitate dissolved in 0.1 N HCl and subsequently neutralized absorbed strongly at 260 mμ, indicating the presence of adenine, and hence the easily labile P of this fraction is almost certainly due to adenosine polyphosphate (ATP and ADP). No difficultly or non-hydrolyzable P esters were precipitated by Ba^{++}, whereas such esters accounted for 62–75%, depending upon pupal age, of the total ethanol insoluble P. The unexpected presence of inorganic P in the ethanol insoluble P derived from pupae half way or more along adult development is probably due, for some unknown reason, to a failure of the preceding Ba^{++} treatment to precipitate the whole of the inorganic P originally present. Thus, the sum of inorganic

P in the Ba^{++} and ethanol precipitates equalled the total inorganic P in the TCA extracts as determined by $CaCl_2$-$Ca(OH)_2$ precipitation.

The ethanol insoluble easily hydrolyzable P most likely consists of glucose-1-P, arginine P, the bulk of the unknown very labile ester and possibly phosphopyruvic acid. Little or no triose P is present, since no inorganic P was liberated by hydrolysis with N NaOH at room temperature. The amount of glucose-1-P is difficult to determine from the available data, as although there was a marked increase in fermentable TRV following 10 min. hydrolysis, chromatography of the resulting hydrolysate showed only a barely perceptible intensification of the pre-existing glucose spot. The very labile ester has been shown to be neither creatine P nor acetyl P, and its extreme lability excludes its identity with the majority of known P esters. Thus, for example, whereas arginine P is 34.7% hydrolyzed by 2.5 N acid at 28° after 135 min. (Meyerhof and Lohmann, '28), under similar conditions the unknown ester is completely hydrolyzed in *c.* 20 min.

Regarding the difficultly and non-hydrolyzable ethanol insoluble P, the present data indicate that glucose and pentose are liberated after prolonged acid hydrolysis. This suggests that glucose-6-P, known to be present in considerable amounts in mammalian tissues, is also accumulated in *Calliphora* pupae, while the source of the pentose is so far unknown.

There is scarcely any information regarding either the constitution or metabolism of phospholipid in insects. The fact that in *Calliphora* the amount of lipid P remained almost constant throughout adult development does not necessarily indicate a constant composition of the phospholipids. It has been shown by Niemierko ('47) that during metamorphosis of the silkworm there is a considerable change in the character of the phospholipids despite only a minor variation in the amount of lipid P.

The close agreement found in estimation of pupal RNA by either nucleic acid P or ribose indicates that the P of this fraction is probably a true measure of RNA. During their isolation of RNA from *C. erythrocephala* larvae, Khouvine and Gregoire ('44) found that DNA formed only 9.2% of the total nucleic acid; if this same proportion obtains in the pupae, it may be calculated that 1.5 gm pupae would yield *c.* 0.05 mg DNA. This small amount would explain why the Dische reaction for DNA was negative in the present experiments, the samples being too small for the desoxypentose of the DNA to give a positive test. The DNA-P bordered upon the limits of error of the methods employed.

Carbohydrates in Calliphora

It has been shown that the carbohydrate content (expressed as glucose) of *C. erythrocephala* has the very high value of over 1.5% of the wet weight, and that the TC is progressively utilized during metamorphosis. This finding, however, is based solely upon the specificity of Dreywood's anthrone reagent for carbohydrate, and the possibility cannot be excluded that non-carbohydrate material of unknown nature may have reacted to yield erroneously high values. Some such explanation may be involved in the anomaly that although acid hydrolysis almost quantitatively converts the initially non-reducing, non-fermentable TC to a reducing and fermentable form, yet very little additional sugar could be demonstrated in the resulting hydrolysate by paper chromatography. However, the latter is in agreement with Frew's ('29) contention that no glycogen, or any polysaccharide yielding glucose after hydrolysis, is present in blow-fly larvae or pupae.

Comparison of the present data with those of Khouvine and Gregoire ('40)

As already indicated, the paper of Khouvine and Gregoire ('40) is of special interest, as these authors also investigated the changes in certain P fractions during metamorphosis of *C. erythrocephala,* but they employed a quite different technique which was briefly as follows: A larval mince, or the juice obtained by squeezing pupae in a press, was thoroughly extracted with boiling ethanol for several hours. An aliquot of the ethanol extract was evaporated to dryness and re-extracted with boiling benzene. The tissue remaining from the ethanol treatment was then extracted with varying concentrations of HCl to give the acid soluble fraction, and the P of the residue was estimated as nucleic acid P. The variation with development of their 4 fractions which, apart from nucleic acid P are only indirectly comparable to those of the present work, are shown in figure 3. The dashed portions of the curves represent changes between the adult just prior to emergence (corresponding to the last analysis of fig. 1), and the free imago. Comparison of figures 1 and 3 shows that the major difference lies in the marked rise and fall in acid

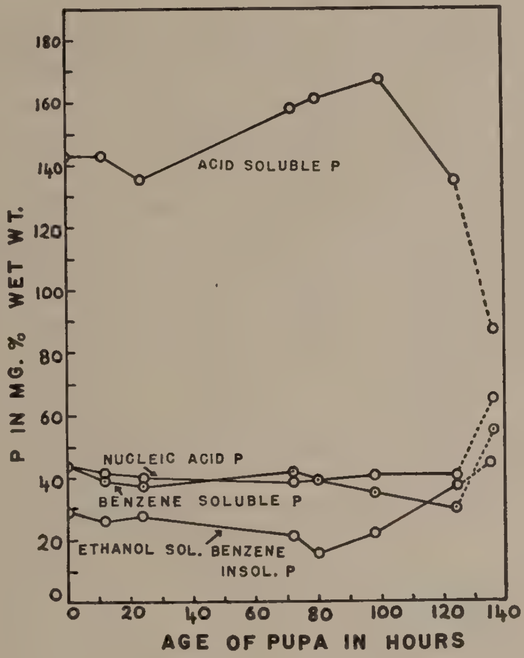

Fig. 3 Variation in the P composition of the blow-fly during metamorphosis. Recalculated from the data of Khouvine and Gregoire ('40). For further details, see text.

soluble P observed by Khouvine and Gregoire. Since the acid soluble P is the most labile of the various fractions, it is the one most likely to reflect the type of method employed.

More difficult to explain, however, are the facts that Khouvine and Gregoire found their pupae had a considerably higher value for total P (260 mg %), and required only 136 hours to complete adult development at 22°C. instead of almost twice this period as is generally the case. This raises the question as to whether the species employed by Khouvine and Gregoire was in fact *C. erythrocephala;* the authenticity of their identification rests upon the classification of a single adult by a competent taxonomist (Khouvine, personal communication), while the large numbers of insects they employed were purchased from a commercial dealer. Since on a commercial scale larvae are generally obtained by allowing exposed pieces of meat to become ''fly-blown,'' it is not unlikely that a mixed population consisting of several genera of meat infesting flies had been used in their studies.

Khouvine and Gregoire's data show that during metamorphosis the pupal water content remains constant at 66% of the wet weight, and there is a progressive decrease in the absolute amount of total P. However, certain anomalies exist in their figures; thus, the loss in over-all pupal weight was at all times considerably greater than the loss in water (e.g., between 0–124 hours 100 pupae lost 3.715 gm in weight while the loss of water was only 2.434 gm). Furthermore, the amount of P expressed on an either dry or wet weight basis remained constant during adult development; it must be concluded, therefore, that since only gases and/or water vapor diffuse through the pupae (as in the cleidoic egg), some volatile P compound was being lost at a rate proportional to the loss in water. The data presented in figure 1 indicate that the major changes in the pupal water content probably occur during the first 30 hours of pupation and not thereafter, and this is in agreement with the findings of Fraenkel and Ruddall ('40).

Changes in P compounds in relation to morphogenesis

During the period of metamorphosis two simultaneous processes occur, namely, the formation of a series of new organs and structures characteristic of the definitive adult, and the progressive degeneration of the old larval tissues through histolysis and phagocytosis. The primary function of the larva is to accumulate sufficient food reserves to supply the energy requirements for adult development, and it is through the breakdown of the larval tissues that these reserves can be utilized, but the mechanism of the autolysis is not yet understood.

From the extensive histological studies of Perez ('10) it is known that whereas some adult organs such as the extremities, sense organs, genitalia, intestine, etc., are entirely new structures, others, e.g. the Malpighian tubules and parts of the nervous system, are largely carried over from the larval to the adult stage with but little change. A few tissues, such as the musculature and hypodermis are composite structures, partly larval, partly new.

Dobzhansky and Poulson ('35) have stated that at no time during the metamorphosis of *Drosophila* is it possible to say that more or less organized tissue exists, since larval histolysis and adult histogenesis proceed concurrently. The P data for *Calliphora* would appear to support this statement; the amount of nucleic acid P, lipid P and acid soluble P remain virtually unchanged during metamorphosis, suggesting that there is no general breakdown of juvenile chemical constituents followed by subsequent resynthesis (as has been previously suggested to explain the U-shaped respiration curve during metamorphosis), but rather a gradual and nicely balanced transformation from the larval to the adult form.

The predominant morphogenic process occurring in *Calliphora* between 144–168 hours of adult development is the formation of the thoracic flight muscles (Agrell, '49), and this accounts for the major part of the increase in respiratory enzymes observed in the whole pupa at this time. The fully formed muscles are rich in easily hydrolyzable Ba^{++} insoluble P and in hexose-P (Baldwin and Needham, '33). (In the thoracic muscles of the closely allied *Phormia regina* most of the easily hydrolyzable P is in fact due to ATP [Levenbook, unpublished].) Referring to figure 2 it can be seen that from about 150 hours there is an increase in difficultly hydrolyzable P, and the formation of easily hydrolyzable P — which had commenced to increase at 80 hours — augments at a slightly faster rate. It is therefore likely that these changes may be correlated with the growth of thoracic muscles.

Probably the most striking feature of the P changes during metamorphosis is the rise and fall in inorganic P and the opposite trend in the 10 min. hydrolyzable P. Heller ('49) has shown that the high concentration of inorganic P in the diapausing pupae of *Deilephila* is due to an accumulation of (probably) potassium phosphate in the gut, the contents of which are converted into an almost solid rod of inorganic P. Heller has made the interesting suggestion that the inorganic P in the gut may be bound and unavailable for metabolism, and this in turn might account for the low respiratory rate. However, in *Calliphora* the larval gut disintegrates almost completely, and hence the inorganic P cannot be bound as in *Deilephila*.

One of the first tissues to be histolyzed during metamorphosis is the voluminous larval fat body; if this contains the considerable quantities of adenosine polyphosphate as found in *Deilephila* (Heller et al., '49), then it may well be that a haemolymph phosphatase similar to that found by Levenbook ('50) in *Gastrophilus* larva blood would rapidly hydrolyze such esters to inorganic P. (In the silkworm Nakamura ['40] found that the level of inorganic P was regulated by the activity of phosphatases.) A similar consideration probably applies to the subsequent histolysis of the larval muscle, and hence the rapid rise of inorganic P and concomitant fall in easily hydrolyzable P could be explained.

It is of interest in the above connection to consider the possibility that the process of oxidative phosphorylation is inhibited during the early stages of metamorphosis. If this were the case, inorganic P would accumulate, and a rapid breakdown of synthesized or stored food material would result. There is evidence from the literature (cf. Needham, '29) that some such catabolic processes, which are important not only for supplying energy, but also for furnishing the new materials for subsequent synthesis (McElroy, '47) do in fact occur in insects. It would be of considerable interest to determine whether during insect metamorphosis there is any change in the usual P : O ratio of 2–4 as found in oxidative phosphorylation by mammalian tissues.

SUMMARY

1. During the metamorphosis of the blow-fly *C. erythrocephala,* no major changes occur in the amounts of acid soluble, lipid or nucleic acid P.

2. The inorganic P increases rapidly during pupation, attains a maximum about half way along adult development, and then declines. The easily hydrolyzable P shows the reverse trend, being high at the beginning and end of pupation, and least at about one-third of the pupal period.

3. No evidence was found for the presence of protein P, or inorganic pyro- or metaphosphate.

4. Difficultly hydrolyzable P is at all times low in concentration, while non-hydrolyzable P esters and an apparently new extremely labile P compound decrease in amount during the period of adult development.

5. Both total carbohydrate (TC) and total reducing value (TRV) are highest at the beginning of pupation and least at the end. Expressed in terms of glucose, the former at all times greatly exceeds the latter, although the TRV approaches the TC level after acid hydrolysis. No glycogen is present in the pupae, and attempts to identify the carbohydrates by means of paper chromatography were only partially successful.

6. The data obtained are compared with those of other workers, and certain correlations between the biochemical results and morphogenesis are tentatively suggested.

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LITERATURE CITED

AGRELL, I. 1949 Localization of some hydrogen activating enzymes in insects during metamorphosis. Nature, *164:* 1039.

ALBAUM, H. G., AND M. KLETZKIN 1948 Adenosine triphosphate from *Drosophila melanogaster*. Arch. Biochem., *16:* 333.

ALLEN, R. J. L. 1940 The estimation of phosphorus. Biochem. J., *34:* 859.

BALDWIN, E., AND D. M. NEEDHAM 1933 The phosphorus distribution in resting fly muscle. J. Physiol., *80:* 221.

CALABY, J. H. 1951 Adenosine triphosphate from insect muscle. Arch. Biochem. Biophys., *31:* 294.

COHN, G., AND I. M. KOLTHOFF 1942 Determination of pyrophosphate. Ind. Eng. Chem. Anal. Ed., *14:* 886.

DISCHE, Z. 1930 Über einige neue charakteristische Farbreactionen der Thymonukleinsaure und eine Mikromethode zur Bestimmung derselben in tierischen Organen mit Hilfe dieser Reactionen. Mikrochemie, *8:* 4.

DOBZHANSKY, T., AND D. F. POULSON 1935 Oxygen consumption of *Drosophila* pupae. II. *D. pseudoobscura*. Z. vergl. Physiol., *22:* 473.

FRAENKEL, G., AND K. M. RUDDALL 1940 A study of the physical and chemical properties of the insect cuticle. Proc. Roy. Soc., *129B:* 1.

FREW, J. G. H. 1929 Studies on the metabolism of insect metamorphosis. Brit. J. Exp. Path., *6:* 205.

HASSETT, C. C. 1951 Transformation of hexosephosphates by the cockroach. Fed. Proc., *10:* 61.

HELLER, J. 1931 Quantitative Studien über die Erbfaktoren der Stoffwechselgrosse bei Schmetterlingspuppen. Biol. Zbl., *51:* 259.

——— 1936 Les composés phosphoriques chez la nymph et la papillon de *Deilephila euphorbiae*. C. R. Soc. de Biol., *121:* 414.

——— 1949 Phosphorus compounds and metabolic rate in insect pupae. Nature, *163:* 952.

HELLER, J., W. SWIECHOWSKA AND ST. KARPIAK 1949 Bilans fosforanowy w czasie przeobrazenia motyla wilczomleczka. Acta Biol. Exp., *25* Suppl. 11: 35.

HELLER, J., ST. KARPIAK AND I. ZUBIKOWA 1950 Inorganic pyrophosphate in insect tissue. Nature, *166:* 952.

JUNI, E., M. D. KAMEN, J. M. REINER AND S. SPIEGELMAN 1948 Turnover and distribution of phosphate compounds in yeast metabolism. Arch. Biochem., *18:* 387.

KHOUVINE, Y., AND M. J. GREGOIRE 1940 Étude biochimique de la métamorphose de *Calliphora erythrocephala*. Bull. Soc. Chim. Biol., *22:* 506.

——— 1944 Constitution de l'acide ribonucleique des larves de *Calliphora erythrocephala*. Bull. Soc. Chim. Biol., *26:* 424.

LEVENBOOK, L. 1950 The composition of horse bot-fly *(Gastrophilus intestinalis)* larva blood. Biochem. J., *47:* 336.

LIPMANN, F., AND L. C. TUTTLE 1945 A specific micromethod for the determination of acyl phosphate. J. Biol. Chem., *159:* 21.

LOHMANN, K. 1928 Über das Vorkommen und den Umsatz von Pyrophosphat in Zellen. Biochem. Z., *202:* 466.

MANN, T. 1944 Studies on the metabolism of mould fungi. 2. Isolation of pyrophosphate and metaphosphate from *Aspergillus niger*. Biochem. J., *38:* 345.

MCELROY, W. D. 1947 The mechanism of inhibition of cellular activity by narcotics. Quart. Rev. Biol., *22:* 25.

MEYERHOF, O., AND K. LOHMANN 1928 Über die naturlichen Guanidinophosphorsauren (Phosphogene) in der quergestreiften Muskulatur. Biochem. Z., *196:* 49.

MILLER, B. F., AND D. D. VAN SLYKE 1936 A direct microtitration method for blood sugar. J. Biol. Chem., *124:* 425.

MORRIS, D. L. 1948 Carbohydrate determination with Dreywood's anthrone reagent. Science, *107:* 254.

NAKAMURA, T. 1940 The phosphorus metabolism during the growth of the animal. The behaviour of various phosphatases and phosphoric acid compounds of *Bombyx mori* L. during growth. Mitt. med. Akad., Kioto, *28:* 387. (Quoted from Chem. Abstracts, 1941, *35:* 3336.)

NEEDHAM, D. M. 1929 The chemical changes during the metamorphosis of insects. Biol. Rev., *4:* 307.

NIEMIERKO, W. 1947 Contribution to the biochemistry of metamorphosis of the silkworm. Acta Biol. Exp., *14:* 151.

NIEMIERKO, S., AND W. NIEMIERKO 1950 Studies on the biochemistry of waxmoth. 6. Metaphosphate in the excreta of *Galleria mellonella*. Acta Biol. Exp., *15:* 111.

PARTRIDGE, S. M. 1948 Filter paper chromatography of sugars. 1. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. Biochem. J., *42:* 238.

PEREZ, C. 1910 Recherches histologiques sur la métamorphose des Muscides. Arch. Zool. Exp. Gen., 4 sér., *5:* 1.

SCHMIDT, G., AND S. J. THANNHAUSER 1945 A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J. Biol. Chem., *161:* 83.

SCHNEIDER, W. C. 1945 Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., *161:* 293.

——— 1946 Phosphorus compounds in animal tissues. III. A comparison of methods for the estimation of nucleic acids. J. Biol. Chem., *164:* 747.

SEVAG, M. G., D. B. LACKMAN AND J. SMOLENS 1938 The isolation of the components of Streptococcal neucleoproteins in serologically active form. J. Biol. Chem., *124:* 425.

UMBREIT, W. W., R. H. BURRIS AND J. F. STAUFFER 1949 Ch. XV in Manometric Techniques and Tissue Metabolism. Burgess Publishing Co., Minneapolis.

VAN SLYKE, D. D. 1917 Studies of acidosis. VII. The determination of β-hydroxybutyric acid, acetoacetic acid and acetone in urine. J. Biol. Chem., *32:* 455.

WALPOLE, G. S. 1911 The direct determination of creatine in pathological urine. J. Physiol., *42:* 301.

THE CLEAVAGE TIMES OF FERTILIZED EGGS OF THE SEA URCHIN, ARBACIA PUNCTULATA, AT HIGH PRESSURES OF NITROGEN, HELIUM, AND NITROUS OXIDE [1,2]

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The hypothesis that nitrogen at increased pressures acts as a narcotic is a plausible one. First suggested by the experiments of Meyer and Hopff ('23) on amphibia, it has received its greatest impetus from the work of Behnke and his coworkers ('35), who attribute the mental and neuromuscular impairment observed in man when breathing air at high pressures, as in deep-sea diving operations, to a nitrogen narcosis. Theoretically, nitrogen falls into line with the indifferent narcotics according to the Meyer-Overton theory of narcosis by virtue of its relatively high lipid-solubility coefficient (Meyer and Hopff, '23; Behnke and Yarbrough, '39). In this connection it seems significant that the substitution of the less lipid-soluble helium for the nitrogen of compressed air enables divers to work with more alertness during compression (Behnke and Yarbrough, '38; Behnke and Willmon, '39).

On the other hand Bean ('45, '50) suggests that the observed effects may primarily be due to an increased carbon dioxide

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[1] This work was aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Rochester (N6onr-241—II).

[2] Preliminary report given at the meetings of the American Physiological Society, Salt Lake City, Utah, September 6–8, 1951. Abstract, Am. J. Physiol., *167*: 793, 794. (1951.)

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tension. Objective evidence for a nitrogen narcosis which is selective has recently been given by Marshall ('51): voluntary movements of intact mice and frogs, and also the brain waves and spinal reflexes of frogs, were found to be abolished by high pressures of nitrogen, while similar exposures administered to the turtle atrium, frog sciatic nerve, and frog nerve-muscle preparation yielded negative results.

If nitrogen acts as a *general* narcotic, its action should be demonstrable upon such cellular activities as are known to be readily affected by indifferent narcotics. To an investigation on this point the cleavage process in the fertilized egg of the sea urchin, Arbacia punctulata, lends itself well. The precision with which Arbacia cleavage is measurable under carefully controlled conditions and the sensitiveness of the process to a great variety of physiologically active agents have long been known (Harvey, '32; Krahl, '50). In the present study the delay in the time required for the first cleavage in Arbacia has been used as the criterion of narcosis during exposure to high pressures of nitrogen.

METHOD

Exposures were made in a small brass pressure chamber of about 1.5 ml capacity which could be sealed with rubber gaskets and screws and which was equipped at top and bottom with heavy glass windows, 3 mm thick, for microscopic observation.[4] To the chamber were attached three stopcocks: one serving as inlet, another as outlet, and the third, for flushing out the flexible copper inlet tube which connected the chamber to the gas cylinder by way of a valve and gauge. A glass container, of similar size to the pressure chamber, was used for the control; its top window of heavy glass could be firmly attached with Lubriseal. The walls of both chambers were lined with filter paper soaked with M/3 sodium carbonate. Since control experiments showed that the chambers contained enough air to provide adequate oxygen for the eggs, the addition of pure oxygen was omitted.

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[4] I am indebted to Dr. W. O. Fenn for the plan of this chamber, which was made by Mr. Arthur Gibson.

A stainless steel water-bath holding 2.8 l of water was used to maintain a constant temperature for the chambers. Supported by legs and with a glass window in its floor, it accommodated a compound microscope, the stage of which had been removed. Working distance for microscopic observation through the water of the bath was obtained by using a 32 mm objective and a 20 × ocular. To avoid distortion by surface ripples the objective was made to descend into a glass well which rested on the tops of the screws which sealed the chamber.

Eggs of Arbacia punctulata which had been strained through gauze, washed three times in filtered sea water, and kept cool before use were brought to the temperature of the water-bath before starting an experiment.[5] Immediately after fertilization, about 120 to 200 (occasionally up to 300) eggs were carefully placed by means of a finely pointed pipette in a small drop on the heavy glass disc which, when inverted, formed the upper window of the pressure chamber. The chamber was then sealed, placed in the water-bath at room temperature or slightly below, and the gas at high pressure was rapidly introduced. The procedures used for the control drop followed the same timing, and precautions were taken to avoid heating or drying of the drops in preparing them. The set-up was completed within 6 to 10 minutes after fertilization.

Although the rapid admission of gas at high pressure would be expected to cause a rise of temperature, the direct contact of the glass window with the water of the bath made it probable that any temperature rise must be very temporary. The consistency of the experimental results bears out the supposition that complicating factors of this type did not occur.

During the cleavage period the experimental and control eggs were examined alternately, and the cleavages in 50 eggs were counted rapidly and multiplied by two to determine per cent cleavage at any one time. By interpolation from the resulting cleavage curves, with per cent cleavage plotted against time, the time of cleavage in 50% of the eggs was determined. This was considered as the cleavage time, and effects on the cleavage rate were obtainable by a comparison of the experimental with the corresponding control value.

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[5] I am very grateful to Dr. Harold F. Blum, and to his co-workers, Mr. Gordon N. Loos and Mr. J. Courtland Robinson, who kindly provided the washed Arbacia eggs and Arbacia sperm used in these experiments.

RESULTS

Control experiments showed that differences of one or two minutes in either direction could be expected between the cleavage times of eggs inside and outside the pressure chamber. This is a larger error than sometimes occurs with this material, but it is probably due to the various manipulations of the procedure.

Fourteen experiments were done with nitrogen at 61 atmospheres and one at 75 atmospheres, no one of which caused a significant alteration in the cleavage time. The entire series is summarized in table 1, from which it will be seen that the cleavage times in the experimentals fell within one or two minutes, slower or faster, of cleavage times in the controls. In terms of percentage difference, only one is more than 3%, and most are much less.

For comparative purposes a parallel series was run with helium since man's breathing of helium-oxygen mixtures at high pressure does not cause the narcotic effects characteristic of nitrogen-oxygen mixtures. The negative results obtained with helium (table 2) at 61 atmospheres were indistinguishable from those with nitrogen with respect to both cleavage curves and cleavage times. In view of the negative findings obtained with nitrogen, this is what might be expected.

Another series using various pressures of nitrous oxide gave evidence that failure of nitrogen at high pressure to delay cleavage is not due to a high resistance to narcosis. Although nitrous oxide is known to be one of the weakest of the narcotizing gases, there is no doubt as to its ability to delay cleavage of the Arbacia egg (table 3). Pressure at 2.3 atmospheres de-

TABLE 1

Minutes required for the first cleavage of fertilized eggs of Arbacia during exposure to nitrogen at high pressure

EXPT. NO.	TEMPERATURE	CONTROLS	EXPERIMENTALS		DIFFERENCE	
		Cleavage time	Nitrogen pressure	Cleavage time	Cleavage time	
	°C.	*minutes*	*atmos.*	*minutes*	*minutes*	*%*
1	18.3–18.8	58½	61	59½	+ 1	+ 1.7
2	18.5–18.6	57½	61	56	— 1½	— 2.6
3	18.5–18.6	57	61	57½	+ ½	+ 0.9
4	18.3–18.7	58	61	59	+ 1	+ 1.7
5	18.3–18.4	57½	61	58½	+ 1	+ 1.7
6	18.0–18.2	58	61	58½	+ ½	+ 0.9
7	18.8–19.0	60	61	61	+ 1	+ 1.7
8	19.0–19.4	57	61	56½	— ½	— 0.9
9	19.0–19.5	58½	61	58	— ½	— 0.9
10	19.2–19.3	56½	62	58½	+ 2	+ 3.5
11	15.2–15.6	90	61	88	— 2	— 2.3
12	16.1–16.7	70½	61	72½	+ 2	+ 2.8
13	16.8–17.0	68	61	68½	+ ½	+ 0.7
14	16.6–16.8	73	61	72½	— ½	— 0.7
15	16.0–16.5	71½	75	72	+ ½	+ 0.7

TABLE 2

Minutes required for the first cleavage of fertilized eggs of Arbacia during exposure to helium at high pressure

EXPT. NO.	TEMPERATURE	CONTROLS	EXPERIMENTALS		DIFFERENCES	
		Cleavage time	Helium pressure	Cleavage time	Cleavage time	
	°C.	*minutes*	*atmos.*	*minutes*	*minutes*	*%*
1	17.7–18.3	66½	61	66	— ½	— 0.8
2	18.6–19.2	59½	61	59	— ½	— 0.9
3	19.4–19.7	61	61	61	0	0
4	18.8–19.3	56½	61	55½	— 1	— 1.8
5	18.0–18.2	66	63	65½	— ½	— 0.8
6	18.3–18.9	60½	61	61	+ ½	+ 0.8
7	18.2	57	62	57	0	0
8	18.2–18.7	53½	61	55½	+ 2	+ 3.7
9	18.8–19.1	58	61	59	+ 1	+ 1.7

layed cleavage 8 minutes in one case and 15½ minutes in another. At 4.8 atmospheres, suppression of cleavage was practically complete. Intermediate pressures often gave abnormal cleavages. When cells showed nuclear, but not cytoplasmic, division as was frequently the case, they were considered as undivided in making counts.

In contrast to the narcosis caused by nitrous oxide, it is significant that nitrogen at 61 atmospheres was found to exert no narcotic effect upon cleavage.

TABLE 3

Minutes required for the first cleavage of fertilized eggs of Arbacia during exposure to nitrous oxide at high pressure

EXPT. NO.	TEMPERATURE	CONTROLS	EXPERIMENTALS		DELAY IN CLEAVAGE TIME
		Cleavage time	Nitrous oxide pressure	Cleavage time	
	°C.	*minutes*	*atmos.*	*minutes*	*minutes*
5	18.1–18.6	58½	2.3	66½	8
8	18.2–18.4	56½	2.3	72	15½
6	15.7–16.0	70	2.9	20% cleavage in 3 hrs.	
4	17.8–18.0	66	3.3	121	55
7	17.7–18.3	68	3.3	1% cleavage[1] in 3 hrs.	
2	17.5–17.8	64	4.1	150	86
3	17.8–18.0	68	4.5	17% cleavage[1] in 2 hrs.	
1	17.5–17.8	63	4.8	2 (?) % cleavage[1] in 5 hrs.	

[1] Cleavages were abnormal.

DISCUSSION

For distinguishing between possible narcotic and hydrostatic effects, experiments with helium which parallel those with nitrogen are of advantage. Since the solubility coefficient of helium is lower than that of nitrogen, any narcotic effects at a given pressure would be expected to be less with helium than with nitrogen, although of course hydrostatic effects would be the same. The negative results obtained with nitrogen as well as with helium indicate the absence of effects of both types.

If one assumes with Behnke that high pressures of nitrogen can produce a narcosis because of lipid solubility, the failure of 61 atmospheres of nitrogen to do this would seem to indicate that insufficient nitrogen pressures were used here. These pressures were far higher, however, than the 10 atmospheres of nitrogen which produced stupefaction in man, the 15 atmospheres estimated for complete human anesthesia (Behnke, Thomson and Motley, '35), and the same pressure as that effective in intact frogs (Marshall, '51). It seems doubtful whether gas pressures much higher than 61 atmospheres could have been used for Arbacia cleavage without encountering hydrostatic effects (Marsland, private communication).

The nitrous oxide series shows the effect of an indifferent, gaseous narcotic and provides data for making an approximate calculation of its molecular concentration in cell lipids which theoretically is required to produce a noticeable degree of narcosis in this particular material. According to Meyer and his associates (Meyer and Hopff, '23; Meyer and Hemmi, '35) this is a relatively constant value for any one biological system, although the threshold concentrations of the various narcotics administered may differ widely. For gases, the equation used is:

$$C_{Lip} = \frac{1}{R} \cdot C \cdot S$$

where C_{Lip} = effective narcotic concentration in cell lipids, in mols per liter,
R = gas constant (0.08206 liter-atm./°/mol × abs. temp.),
C = effective pressure of administered gas, in atmospheres,
S = solubility coefficient (ml/ml olive oil).

Solving for nitrous oxide, where
R = 23.9 liter-atm. at 18°C.,
C = 2.3 atmospheres, at lowest effective concentration studied here,
S = 1.5 (S at 17°C., from Meyer and Gottlieb-Billroth, '21).

$$C_{Lip} = \frac{1}{23.9} \times 2.3 \times 1.5$$

$$C_{Lip} = 0.144 \text{ mols/liter.}$$

This is not far from Meyer and Hopff's value of 1.18 for narcosis of frogs with nitrogen, and of Marshall's value of 0.117 and 0.112 for inhibition of frog brain waves with nitrous oxide and nitrogen respectively. Assuming that C_{Lip} is constant for any one biological system, this value of 0.144 is used for calculating the theoretical pressure of nitrogen necessary to produce a similar delay in cleavage. S for nitrogen $= 0.05$ (Vernon, '07).

$$0.144 = \frac{1}{23.9} \times C \times 0.05$$

$C = 68.8$ atm., calculated effective pressure of nitrogen.

Considering the approximate nature of these figures, this value of 68.8 atmospheres is not far from the 61 atmospheres used in these experiments, and it is less than the 75 atmospheres employed in one of them. It seems probable that if the lipid-soluble property of nitrogen could be effective in delaying cleavage it would have been evident to some small extent at the pressures used in these experiments. The evidence here presented therefore does not bear out the hypothesis of a *general* narcotic action of nitrogen at high pressure.

SUMMARY

1. The hypothesis that nitrogen at high pressure exerts a general narcotic action has been tested by subjecting the fertilized eggs of the sea urchin, Arbacia punctulata, to nitrogen pressures of 61 atmospheres, in the presence of air at atmospheric pressure. A series with helium at the same pressure was run for comparison.

2. Neither nitrogen nor helium at 61 atmospheres affected the time of appearance of the first cleavage.

3. The weak narcotic, nitrous oxide, caused a definite delay of cleavage at pressures as low as 2.3 atmospheres.

It is a pleasure to acknowledge my indebtedness to Dr. Wallace O. Fenn for his suggestion of the problem of nitrogen narcosis and for his continued interest in it.

LITERATURE CITED

BEAN, J. W. 1945 Effects of oxygen at increased pressure. Physiol. Rev., *25:* 1–147.

——— 1950 Tensional changes of alveolar gas in reactions to rapid compression and decompression and question of nitrogen narcosis. Am. J. Physiol., *161:* 417–425.

BEHNKE, A. R., R. M. THOMSON AND E. P. MOTLEY 1935 The psychologic effects from breathing air at 4 atmospheres pressure. Am. J. Physiol., *112:* 554–558.

BEHNKE, A. R., AND T. L. WILLMON 1939 U.S.S. *Squalus,* Medical aspects of the rescue and salvage operations and the use of oxygen in deep-sea diving. 1939 U.S. Naval Med. Bull., *37:* 629–640.

BEHNKE, A. R., AND O. D. YARBROUGH 1938 Physiologic studies of helium. U.S. Naval Med. Bull., *36:* 542–558.

——— 1939 Respiratory resistance, oil-water solubility, and mental effects of argon, compared with helium and nitrogen. Am. J. Physiol., *126:* 409–415.

HARVEY, E. N. 1932 Physical and chemical constants of the egg of the sea urchin Arbacia punctulata. Biol. Bull., *62:* 141–154.

KRAHL, M. E. 1950 Metabolic activities and cleavage of eggs of the sea urchin, Arbacia punctulata, a review, 1932–1949. Biol. Bull., *98:* 175–217.

MARSHALL, J. M. 1951 Nitrogen narcosis in frogs and mice. Am. J. Physiol., *166:* 699–711.

MARSLAND, D. Private communication.

MEYER, K. H., AND H. HEMMI 1935 Beiträge zur Theorie der Narkose. III. Biochem. Ztschr., *277:* 39–71.

MEYER, K. H., AND H. HOPFF 1923 Theorie der Narkose durch Inhalationsanästhetika. II. Narkose durch indifferente Gase unter Druck. Ztschr. f. physiol. Chem., *126:* 281–298.

MEYER, K. H., AND H. GOTTLIEB-BILLROTH 1921 Theorie der Narkose durch Inhalationsanästhetika. Ztschr. f. physiol. Chem., *112:* 55–79.

VERNON, H. M. 1907 The solubility of air in fats, and its relation to caisson disease. Proc. Roy. Soc. Lond., *B 79:* 366–371.

RECOVERY OF X-IRRADIATED BACTERIA AT SUBOPTIMAL INCUBATION TEMPERATURES[1]

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FOUR FIGURES

The observation of recovery of living cells from the effect of ionizing radiations has been reported repeatedly. The literature concerning this effect has been reviewed by Lea ('47) and more recently by Gray ('52) and Allen et al. ('51). Several techniques have been employed to demonstrate recovery in living organisms, e.g., comparison of the efficiency of single and fractionated doses of a radiation or comparison of the efficiency of a radiation applied at high and low intensities. The use of these techniques has yielded information concerning the recovery which occurs during the course of radiation exposure. A review of the literature (Lea, '47) indicates that bacteria irradiated in aqueous suspensions do not recover during the exposure to ionizing radiations. Some types of cells are able to recover from the effects of ionizing radiations if maintained in the cold or in a starved condition after irradiation (Strangeways and Fell, '27; Spear and Glücksmann, '39; Cook, '39; Allen et al., '51). In some of the cases reported, however, apparent recovery was in fact merely a postponement of the radiation effect, which was shown to take its normal course when active metabolism and growth were resumed. Latarjet ('43) showed that holding bacterial cells on nutrient agar at refrigerator temperatures

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[1] Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

(0 to 6°C.) did not increase the number of colony-forming organisms over that obtained by incubation at 37°C.

Noteworthy among reported investigations concerning recovery are those of Henshaw ('40) and Lamarque and Gros ('45) which give some information about the mechanism of the recovery process. The results of both investigators seem to indicate that recovery from x-ray effects occurs only under conditions in which metabolism can occur.

We have investigated the effect of incubating x-irradiated *Escherichia coli* B/r at temperatures below those which are optimal for growth (6–30°C.). It was found that, although there was no increase in the number of viable organisms with incubation at 6°C. over that obtained at 37°C., significant increases in survival were obtained at all intermediate temperatures (12–30°C.).

MATERIAL AND METHODS

Escherichia coli B/r was grown in Difco nutrient broth (0.8%) under constant aeration at 37°C. The preparation of the samples for irradiation as well as the techniques for exposure were similar to those described by Stapleton, Billen, and Hollaender ('52). All suspensions were prepared in M/15 phosphate buffer (pH 6.8) and irradiated in equilibrium with atmospheric air at ice-bath temperature. Immediately after irradiation, the suspensions were appropriately diluted in sterile water and 0.05 ml of the final dilutions spread on the surface of nutrient agar plates. Triplicate platings were made. Prior to seeding, the plates were cooled in incubators at the desired temperatures, diluted suspensions plated rapidly, and the plates returned to the proper incubators where they were held for 24 hours. The plates were then removed to a 37°C. incubator and, after an additional 24-hour incubation period, the relative survivals at the various holding temperatures were determined by colony counts. The 24-hour holding period at the various temperatures was determined previously to be substantially longer than that required for maximal survival at any of the temperatures used. Nonirradiated control samples were prepared and treated in the same manner.

In those experiments in which liquid media were utilized for studying recovery, 1 ml of the irradiated suspension was added to 9 ml of the medium, and incubated under aeration in constant-temperature water baths at the various temperatures. For convenience, control samples were so diluted that the initial number of colony-forming organisms were the same in control and irradiated samples. Aliquots were removed at various times after inoculation, diluted in sterile water, and surface plated on nutrient agar. The number of viable cells was determined by plate count after 24 hours' incubation at 37°C.

EXPERIMENTAL RESULTS

Effect of postirradiation incubation temperature on survival

In this portion of the investigation, irradiated cells were spread on nutrient agar plates and held for 24 hours at the various temperatures. The plating technique was considered most applicable to this study, since any detected increase in survival during incubation at the experimental temperatures could result only from an increase in ability of existing cells to form colonies. Figure 1 is a plot of surviving fraction of irradiated cells as a function of the temperature of incubation. The change in survival with temperature at three dose levels is shown. It is apparent from the data that, in agreement with Latarjet ('43) there is no significant difference in survival at a particular dose, for cells incubated at 6° and 37°C. However, our data indicate that, at all intermediate temperatures studied, the number of colony-forming organisms is significantly increased, passing through a maximum at about 18°C. It is of interest that control cells show no such variation in viability with temperature, the survival at all the temperatures used being a constant within the error of experimentation. It should be noted that the ratio

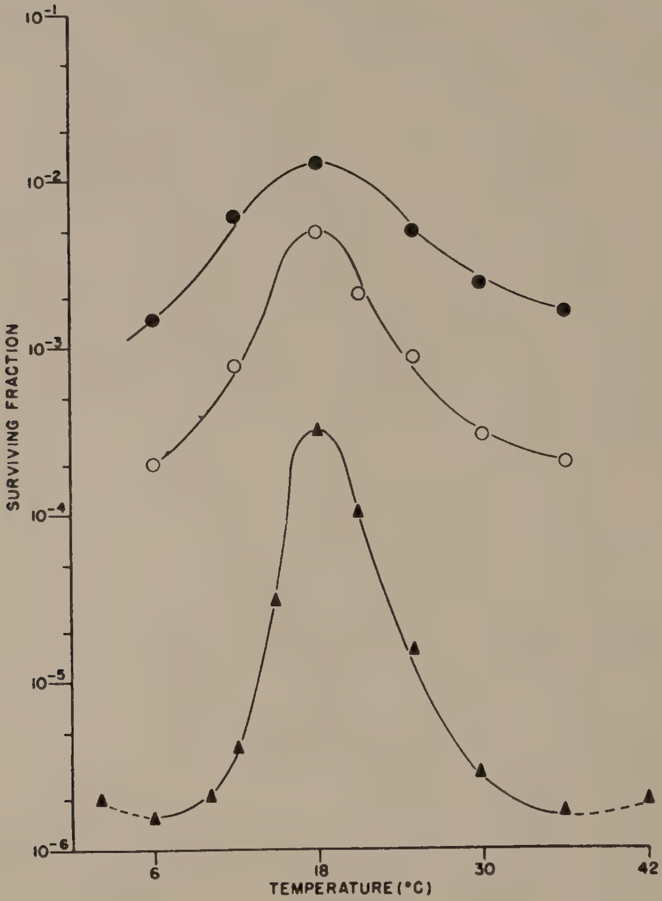

Fig. 1 Survival of *E. coli* B/r at several x-ray doses as a function of incubation temperature.

● — 40 kr ○ — 60 kr ▲ — 80 kr

of the surviving fraction at 18°C. to that at 37°C. is an increasing function of x-ray dose. This will be discussed in a later section.

Rate of recovery of irradiated cells at various temperatures

In order to obtain information concerning the mechanism of recovery, it was necessary to investigate the rate at which this process proceeds at the different temperatures (6°–30°C.). Since in these experiments we measure the amount of recovery at any temperature relative to that obtained at 37°C., it would seem that removing the plates from a lower temperature, rapidly warming them to 37°C., and maintaining them thereafter at this temperature should give a quantitative measure of the relative recovery which took place at the experimental temperature. A large number of duplicate sets of plates were preincubated at the various temperatures, spread with 0.05-ml aliquots of the diluted irradiated suspensions, and then returned to the proper incubators. After different time intervals sets of plates were removed from the lower temperature incubators, warmed rapidly to 37°C. in a 50°C. incubator (15–20 minutes required), then incubated at 37°C. for 24 hours. At the end of that period the colonies were counted to determine the relative survivals. Control plates were prepared and treated similarly. Plates left at temperatures from 15°–30°C. for as long as 96 hours were countable without further incubation at 37°C. yielding counts similar to those held for 12 hours at the lower temperature then counted after 24 hours' incubation at 37°C. The results of several experiments are shown in figure 2. The cells in all cases received 70 kr of x rays. Surviving fraction (N/N_0) is plotted as a function of the holding time at the temperatures indicated. It is clear from these data that the increase in survival is initially an exponential function of the holding time at a particular temperature. The rate of recovery, as measured by the initial slopes of the curves, increases with increasing temperature. A finding of utmost importance is that, although the rate of recovery at temperatures above 18°C. increases with increasing temperature, the final survival (survival at optimum holding time) decreases. It should be noted that the optimum holding time at any of the temperatures studied is less than 24 hours.

Incubation of irradiated cells in nutrient broth at the various temperatures gave essentially the same recovery rates as those obtained on solid medium at each investigated tem-

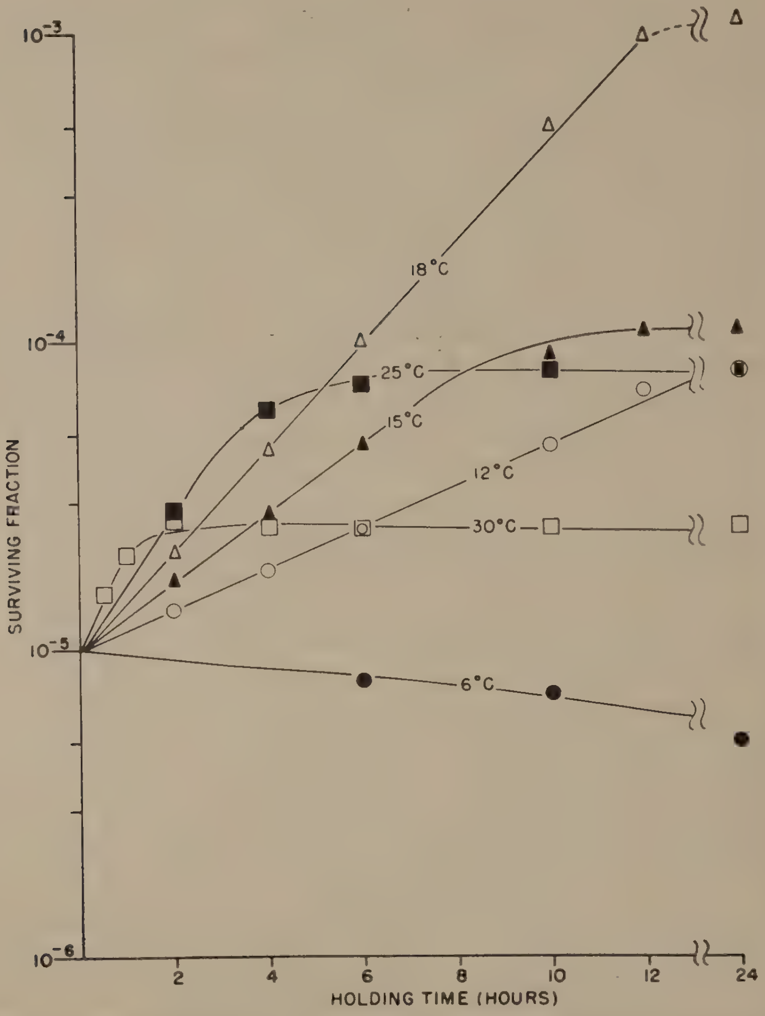

Fig. 2 Rates of recovery of *E. coli* B/r at various temperatures after 70 kr of x rays.

perature. Unirradiated control cells, at any temperature studied, showed a lag period prior to increase in cell numbers, the duration of this lag period being temperature dependent ($Q_{10} = \sim 2.4$). On the other hand, there was an increase in number of viable cells in cultures of irradiated cells without a lag at all temperatures below 37°C. Moreover, the initial rate of increase in colony-forming cells in cultures of irradiated cells parallels the rate of growth of irradiated and nonirradiated cells at the various temperatures.

Preliminary experiments in which media other than nutrient broth were utilized indicated that irradiated cells are unable to recover in an organic salts-glucose medium which will sustain growth and cell division in control cells. This finding suggests the existence of a special nutritive requirement for the recovery process. The problem of isolating such a factor is being investigated at present; the results will be reported in a subsequent publication.

Survival at various temperatures as a function of x-ray dose

The curves shown in figure 1 suggested that the ratio of surviving fraction at the optimum temperature (18°C.) to that at 37°C. increased with increasing x-ray dose. This finding is demonstrated more clearly by figure 3. The surviving fractions at 18° and 37°C. are plotted as functions of x-ray dose. Within experimental error, the curves are exponential over the range of doses used. The apparent effect of holding irradiated cells on nutrient agar at the lower temperature is a systematic reduction of the efficiency of the radiation per unit dose. In the expression for the survival fraction as a function of dose.

$$\ln N/N_0 = -kd$$

where d equals the dose and k, the inactivation coefficient

$$k_{37}/k_{18} = 1.5.$$

Recovery of other strains of Escherichia coli

It was observed that various strains of *E. coli* have different sensitivities to x radiation under identical cultural and treatment conditions. Three strains tested, B/r, Crooks, and Texas showed increasing resistances to x rays in the order mentioned, when postirradiation incubation was carried out at 37°C. It seemed desirable to test the relative recoverability of irradiated cells of the three strains. Cells of these strains cultured, were harvested, irradiated, and plated in an identical manner at the various temperatures. As shown in figure 4, all three strains showed the recovery effect, but differed from one another both in the magnitude of the effect as well as in the optimum temperature for recovery. This

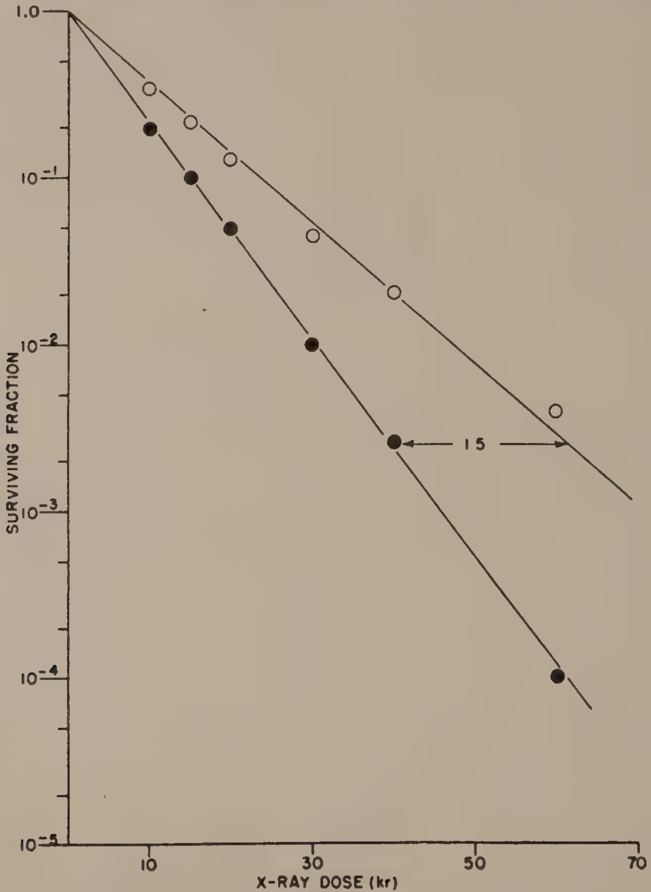

Fig. 3 Survival of *E. coli* B/r at various x-ray doses.
○ — holding at 18°C. ● — holding at 37°C.

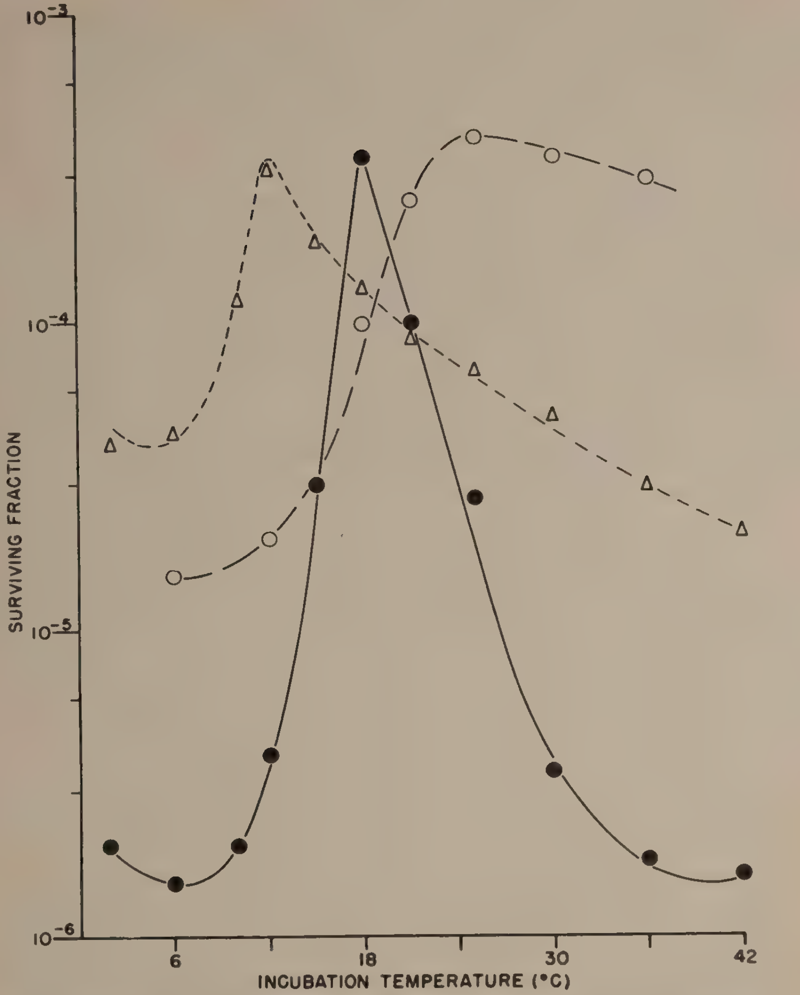

Fig. 4 Survival of three strains of *E. coli* at 80 kr at various temperatures.
△ — Crooks ● — B/r ○ — Texas

figure also shows that postirradiation incubation of cells of the three strains at 37°C. gives survival ratios indicative of a wide variation in sensitivity. It should be noted, however, that the surviving fraction for the various strains at a constant x-ray dose, is essentially the same when each strain is incubated at its optimum recovery temperature. This finding suggests that strain differences in radiosensitivity may be a reflection of the amount of recovery which can occur under the conditions of postirradiation treatment.

DISCUSSION

The finding that, under proper cultural conditions, bacterial cells are able to overcome to some extent the lethal effects of ionizing radiations is not wholly unexpected, since a variety of types of living cells have been shown to possess this property (Lea, '47; Allen et al., '51; Gray, '52). Hollaender and Duggar ('38) found that ultraviolet-irradiated bacteria cultured under conditions suboptimal for growth showed an "apparent initial increase in colony forming cells." Their data suggest that a process similar to recovery as described in this paper may have been operative. The real importance of the findings concerning bacterial recovery from the lethal effects of x rays reported in this paper lies in the possibility that further study might result in a better understanding of the physiological nature of the process. Although the data reported permit little more than speculation concerning the nature of recovery, they do tend to minimize some suggested mechanisms: (1) Recovery cannot be explained as a simple, nonphysiological decay of a toxic product of the radiation, since cells held at the same temperature in inorganic salts-glucose medium did not recover although incubated for a length of time sufficient for maximal recovery in a more complete medium (nutrient broth). This finding suggests, moreover, that there might be a special nutritive requirement for the recovery process, since growth and cell division can occur in the minimal medium. (2) Our data seem to eliminate the possibility that recovery is due to a stimulation of cell division (i.e., without a normal lag period), since such a process could not account for any increase in colony-forming organisms on solid media with increasing incubation time at any temperature. (3) The similarity of the recovery rates obtained by incubating irradiated cells at reduced temperatures in liquid media or on solid media indicates that the same process occurs on either type of medium.

The curves relating survival to x-ray dose at temperatures for minimal and maximal recovery, 37° and 18°C. respectively (fig. 3), show a systematic reduction of the lethal effect per unit dose brought about by reduction of the incubation temperature. Photoreactivation of bacteria after ultraviolet irradiation (Kelner, '49; Novick and Szilard, '49) brings about a similar alteration in lethal effectiveness of a radiation. A currently accepted explanation for photoreactivation is the partial destruction of an ultraviolet-produced toxic substance by posttreatment with visible or near-visible light. An apparent difference in photoreactivation and recovery, as discussed here, is that recovery seems to be brought about by a physiological process.

It can be postulated that the ability of irradiated bacteria to survive a given dose of x rays is dependent to some extent on the relative rates of two competitive metabolic processes. One of these processes has the characteristics of synthesis, and is prevented from a more complete repair of the irradiated cells at temperatures above 18°C. by the intervention of a destructive process. A somewhat similar hypothesis has been suggested by Hinshelwood ('46) to explain the influence of temperature on growth of bacteria. The nature of the data obtained for survival at a constant dose as a function of temperature (fig. 1) and also for the rates of recovery at various temperatures as a function of time (fig. 2) are consistent with this hypothesis. The nature of the destructive process is unknown at present, but it appears that radiations might induce an increased thermolability of some systems in living cells. This suggestion has been made by Clark ('38) and more recently by Giese and Heath ('48) to explain increased heat sensitivity of irradiated cells. We might assume from the experiments of Clark ('38) that proteins can be denatured at lower temperatures after sensitization by irradiation.

Why different strains of the same species of bacteria show different temperature optima for recovery is not known, but the finding suggests that a more thorough examination of the temperature relationships for other physiological processes in these strains might give valuable information as to the mechanism of inactivation of bacterial cells by ionizing radiations.

SUMMARY

1. *Escherichia coli* can partially recover from the lethal effects of x rays if incubated in the presence of available nutrient at temperatures below those which are normal for growth.

2. Recovery has been shown to be neither a nonphysiological decay of a radiation-produced toxic substance, nor a stimulation of early cell division in irradiated cells.

3. Cells recover similarly on complete media, whether liquid or solid.

4. Irradiated bacteria are not able to recover in a minimal medium which will sustain growth and cell division, suggesting a special nutritive requirement for recovery.

5. Strain differences have been observed in regard to the optimal temperature for recovery, as well as to the magnitude of the effect.

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LITERATURE CITED

Allen, B. M., Ole Arne Schjeide and L. B. Hochwald 1951 The influence of temperature upon the destruction of hematopoietic cells of tadpoles by x-irradiation. J. Cell. and Comp. Physiol., *38:* 69–82.

Clark, J. H. 1938 The temperature coefficient of the effect of radiation on proteins and its relation to injury in the living cell. Am. J. Roentgenol. Radium Therapy, *40:* 501–508.

Cook, E. V. 1939 Influence of low temperature on recovery from roentgen rays. Radiology, *32:* 289–293.

Giese, A. C., and H. D. Heath 1948 Sensitization to heat by x-rays. J. Gen. Physiol., *31:* 249–258.

Glücksmann, A., and F. G. Spear 1939 The effect of gamma radiation on cells *in vivo*. Brit. J. Radiol., *12:* 486–498.

Gray, L. H. 1952 Biological actions of ionizing radiations. In Progress in Biophysics and Biophysical Chemistry. Academic Press, Inc., vol. 2, pp. 240–305.

Henshaw, P. S. 1940 Action of x-rays on gametes of *Arbacia punctulata*. IV. The influence of low temperature on recovery from roentgen-ray effects in the eggs. Am. J. Roentgenol. Radium Therapy, *43:* 921–922.

Hinshelwood, C. N. 1946 The Chemical Kinetics of the Bacterial Cell. Oxford University Press, London.

Hollaender, Alexander, and B. M. Duggar 1938 The effects of sublethal doses of monochromatic ultraviolet radiation on the growth properties of bacteria. J. Bact., *36:* 17–37.

Kelner, A. 1949 Photoreactivation of ultraviolet-irradiated *Escherichia coli* with special reference to the dose-reduction principle and to ultraviolet induced mutation. J. Bact., *58:* 511–522.

Lamarque, J. P., and C. Gros 1945 The eggs of the *Bombyx-Mori* as material for radiobiological research. Brit. J. Radiol., *18:* 293–296.

Latarjet, R. 1943 Action du froid sur la réparation de radiolésions chez une levure et chez une bactérie. Compt. rend., *217:* 186–188.

Lea, D. E. 1947 Actions of Radiations on Living Cells. The Macmillan Co., New York.

Novick, A., and L. Szilard 1949 Experiments on light-reactivation of ultraviolet inactivated bacteria. Proc. Natl. Acad. Sci. U. S., *35:* 591–600.

Stapleton, G. E., Daniel Billen and Alexander Hollaender 1952 The role of enzymatic oxygen removal in chemical protection against X-ray inactivation of bacteria. J. Bact., *63:* 805–811.

Strangeways, T. S. P., and H. B. Fell 1927 A study of the direct and indirect action of x rays upon the tissues of the embryonic fowl. Proc. Roy. Soc. (London) B., *102:* 9–29.

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